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UNIVERSITY OF ALBERTA

Determining the Optimal Conditions For Hypothermic Rat Pancreas Preservation

by

Jonathan Robert Todd Lakey

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

Department of Zoology

Edmonton, ALBERTA

Fall, 1990

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FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled DETERMINING THE OPTIMAL CONDITIONS FOR HYPOTHERMIC RAT PANCREAS PRESERVATION submitted by Jonathan Robert Todd Lakey in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE.

(Supervisor) .....
Dr. Lawrence Wang

(Committee Member) .....
Dr. Ray Rajotte

(Committee Member) .....
Dr. Michael Wolowyk

(Examiner) .....
Dr. Hugh Clifford

Date: September 24, 1990

DEDICATION

This text is dedicated to my parents, Dr. William and Shirley Lakey, who have always provided inspiration, encouragement and the desire to accept challenges and to strive for excellence.

ABSTRACT

Transplantation of islets of Langerhans as a means of compensating for the lack of endogenous functional beta cells is becoming a viable alternative in the treatment of diabetes mellitus. The protocols for optimal pancreas storage prior to the isolation of the islets, however, have not yet been fully established. The scientific studies have rendered uncertainty as to whether the preciously available pancreas has been maximally protected. The present study was undertaken to first establish in an rat model the optimal temperature and preservation solution for pancreas storage prior to islet isolation.

Rat pancreases were hypothermically (4°C) preserved in either University of Wisconsin (UW) or Euro-Collins preservation solution to compare their efficacy. Following a 24 hour preservation period, islets were isolated by collagenase digestion and Ficoll gradient separation. The superiority of UW solution was clearly evident ($p < 0.001$) in total islet yields (318 vs less than 10). Utilization of the UW solution in rat pancreas preservation would clearly extend the hypothermic preservation time prior to islet harvest.

Pancreatic ductal distension via the common bile duct using either UW or Euro-Collins solution was determined to be superior to in situ vascular flushing of the pancreas via the abdominal aorta when comparing islet cell yields in both fresh

and 24 hour hypothermic preserved pancreases.

To evaluate the effect of preservation temperature on islet yield and subsequent islet function, several hypothermic temperatures (4, 7, 10, and 15°C) were studied. Following 24 hour hypothermic storage, the recovery of islets was poorest in the 15°C preservation group, followed by the 4°C group and significantly higher in the 7 and 10°C groups ($p < 0.05$). Islet function, as measured by insulin output following hyperglycemic challenge by glucose perifusion, was reduced ($p < 0.0001$) in all preservation groups. The mean insulin secretion rate (MISR) was lowest in the 4°C group, highest in the 7°C group and intermediate in the 10°C group. The islet preservation index (IPI) was more than double in the 7 and 10°C preservation groups as compared to the 4°C group ($p < 0.001$). Significant improvements in islet yield and function were achieved when the rat pancreas was preserved at 7 and 10°C rather than at 4°C.

Comparison of preserving islets which were freshly isolated, versus those which were isolated after the whole pancreas preservation, showed a similar qualitative insulin response after cold storage at 7°C for 24 hours. However, the regulated insulin secretion (MISR) was significantly better ($p < 0.0001$) in the isolated islet preservation group than in the islets isolated after whole pancreas preservation.

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LITERATURE REVIEW[1]

Diabetes mellitus is a chronic disorder of glucose and carbohydrate metabolism (1). This devastating disease is characterized by various physiological abnormalities, most notably, prolonged hyperglycemia (1, 2). Diabetic individuals experience a digression from normal blood glucose levels, a consequence of defective biosynthesis, release or utilization of the pancreatic hormone, insulin. The prolonged exposure to hyperglycemia can have a profound and devastating effect on the diabetic's physiological state (2, 3). Metabolic disturbances brought on by prolonged hyperglycemia include nephropathy, retinopathy, neuropathy and microangiopathy (2-4). These chronic complications manifested in diabetic persons lead to diabetics being: ten times more likely to experience coronary artery disease with atherosclerosis appearing at an early age, four to six times more likely to develop cataracts, twenty-five times more likely to develop blindness, seventeen times more likely to develop kidney dysfunction and in one half of all diabetics, renal failure

[1] A portion of this work has been submitted for publication: Lakey JRT, Wang LCH, Rajotte RV. Transplantation: 1990.

as a result of nodular glomerulosclerosis, sixteen times more likely to develop gangrene of the limbs leading to non-traumatic amputations, and sensory and autonomic changes due to neuropathic deterioration (3, 5, 6). Consequently, the life expectancy of the nearly eleven million North Americans afflicted with diabetes is only two-thirds that of the normal population (4). Diabetes is the fifth leading cause of death in North America (15.2 deaths per year per 100,000 deaths) (4, 6).

Diabetes can be classified as primary or secondary. Type I, primary juvenile diabetes or insulin-dependent diabetes mellitus (IDDM), is characterized by the abrupt onset of symptoms, onset primarily prior to age of 25 years and most notably absolute insulin deficiency (2, 6). Type II primary diabetes or non-insulin-dependent diabetes (NIDDM) occurs predominately in mid or late life and is prevalent in obese individuals (7). Secondary diabetes mellitus can result from chronic pancreatitis, hormonal abnormalities, and certain genetic syndromes (7).

Type II diabetes is often related to an insufficient release of insulin from the beta cell of the pancreas to meet the tissue needs and to maintain blood glucose homeostasis. Treatment of type II diabetes is normally managed with a balance of energy expenditure and strict dietary control (2).

With type I diabetes, the prevention of wide fluctuations of hypoglycemia and hyperglycemia are controlled through balancing dietary intake and energy expenditure with exogenous insulin therapy (2). The exogenous administration of insulin is required in order to maintain the fasting plasma glucose concentrations within the normal narrow range (2). However, stringent control and intensive multiple daily insulin injections are needed to maintain normoglycemia (8). The development of portable blood glucose monitoring units, "open loop" and "closed loop" continuous insulin injection systems have provided a means to better monitor blood glucose levels (8-10). However, the lack of complete success of these systems and the problems associated with infection, chronic peripheral hyperinsulinemia and the possibility of inadvertent hypoglycemia have drawn researchers to focus their attention on the transplantation of viable islets as a means of compensating for the lack of endogenous insulin-producing cells (11, 12).

The most physiologically appropriate and logical alternative to exogenous insulin therapy is "total endocrine replacement therapy" via transplantation of the intact pancreas or transplantation of a free graft of pancreatic tissue (13). Transplantation of the intact pancreas involves either the vascularized whole organ or a segmental portion of

the pancreas. Whereas, transplantation of a free graft of pancreatic tissue involves either dispersed pancreatic tissue fragments or isolated islets of Langerhans. Early efforts to alleviate diabetes through pancreas transplantation were attempted soon after the work of Von Mering and Minkowski, who in 1889, were first to show that hyperglycemia developed following the complete removal of the pancreas (14, 15). Drs. Williams and Harsant were first to attempt a clinical transplantation by a subcutaneous implantation of pieces of a sheep pancreas in 1893 (15, 16). However, this initial attempt, as well as other early pancreatic allografts, were unsuccessful on a long term basis (13, 15). The lack of success was mainly due to the technical complications associated with the procedure, improper control or management of the exocrine secretion as well as the pancreatic graft being extremely vulnerable to tissue rejection (13, 17, 18).

A therapeutic approach to managing diabetes was favoured following the introduction of insulin in early 1922 (19). Frederick Banting and his assistant Charles Best, working in the physiology lab of Dr. J.J. MacLeod at the University of Toronto, hypothesized that the secretions from the exocrine portion of the pancreas were involved in preventing successful extraction of the substance from the islets (19). Ligation of the pancreatic duct induced the selective degradation of

the exocrine portion of the pancreas (19). Banting and Best were then able to successfully isolate the secretion from the islets in the atrophied pancreatic tissue. On July 30, 1921 this extract, first termed "isletin," was able to control the blood glucose level in a pancrectectomized dog (19, 20). Purification of the hormone, insulin, by Dr. J.B. Collip helped improve the first clinical trials in early 1922 (20). Banting and MacLeod were rewarded with the Noble Prize in 1922 for their keen insight into the discovery of insulin (20). Administration of insulin appeared to be a cure for the acute and fatal complications of diabetes. It was Banting, however, who said, "Insulin is a treatment not a cure" (15, 20). This statement soon become more apparent, because, with the increase in life expectancy of a diabetic individual on exogenous insulin therapy, the late chronic complications of the disease were soon manifested. It was evident that transplantation of functional islets was the only method to achieve totally normal glucose metabolism (13).

With the development of new surgical techniques and the success of newly developed immunosuppressive agents such as cyclosporin-A, interest was renewed in pancreatic tissue transplantation (15). One of the major obstacles to the success of pancreas transplants, was the management of the digestive enzymes secreted by the exocrine portion of the

pancreas. Anastomosis of the pancreatic duct in the transplanted pancreas to the ureter (21, 22), jejunum (23) and free drainage into the peritoneal cavity (24) were attempts made to prevent the autodigestion of the graft and to prevent vascular thrombosis leading to the loss of the endocrine function (21, 25). Blockage of the pancreatic duct with neoprene (26) or ligation of the pancreatic duct (27) have also been attempted to manage or control the exocrine drainage (13, 21). Since the first clinical attempt in December of 1966, by Kelly, Lillehei and associates (28), to June 30, 1989 there have been 2004 clinical whole pancreas transplantations performed (29, 30). Unfortunately, the one year graft survival rate (insulin-independent) has been only 46% (29, 30). Technical difficulties, requirements for, and complications of immunosuppression therapy, and the limited storage capacity of the whole pancreas, have prevented the large scale application of pancreas transplantation (13, 21). These obstacles led researchers to explore the transplantation of isolated islets of Langerhans.

The option of transplanting only the endocrine portion of the pancreas has theoretical advantages. Islet transplantation is technically easier than whole pancreas transplantation and there are no problems of managing the exocrine secretions (13). It was hoped that by transplanting

a small volume of pure islets there would be a reduced immunogenic response. However, islet grafts have been shown to be as immunogenic as the whole pancreas grafts (31, 32). Alteration or modulation of isolated islets to reduce their immunogenicity have recently been documented (33, 34).

Bensley, in 1911, was the first scientist to extract the islets of Langerhans via hand picking the islets following staining of the pancreatic tissue with neutral red stain (35, 36). Hellerstrom developed a method of free-hand micro-dissection, mechanically removing the islets from the surrounding tissue via a dissecting microscope (37). However, this was labour intensive and islets were not viable following isolation. Moskakowski, in 1965, introduced the use of the enzyme collagenase, a compound produced by the bacteria Clostridia histolytium, to isolate functionally viable islets of Langerhans from the surrounding tissue of the guinea pig pancreas (38). The enzyme cleaves the peptide bonds in the collagen fibers of the tissue and allowed the tissue dissociation which facilitates the separation of the islets from the unwanted acinar tissue (38).

Lacy and Kostianovsky distended the pancreas by retrograde perfusion via the common bile duct with Hanks' balanced salt solution (HBSS) (39). The intralobular distension provides a means to mechanically separate the

islets from the exocrine tissue prior to the biochemical separation via collagenase digestion, enabling high numbers of islets (200 - 300 islets from a single rat pancreas) to be recovered (39). Lacy and Kostianovsky were also the first to attempt separation of the islets from the exocrine tissue by centrifugation in a discontinuous sucrose gradient (39). Differences in the density of islets (1.075 - 1.085 g/cm^3) as compared to to the density of exocrine tissue fragments (1.125 - 1.141 g/cm^3) facilitate a rapid and effective means to separate pure isolated islets from the unwanted exocrine tissue (40). The hyperosmolar sucrose solution had been shown to affect the _in vitro_ response of the isolated islets and was replaced with Ficoll, a high molecular weight (400,000 dalton) polymer of sucrose, by Lindell _et al._, in 1969 (41). Sharp _et al._, (1973) dialysed the Ficoll prior to centrifugation to remove contaminants from the Ficoll solution and obtained better islet recovery (42). Islets isolated with the collagenase digestion and Ficoll purification techniques responded to the glucose stimulation similar to results found in the manual technique of hand picking the islets (43, 44). A reflected green light technique was developed to further assist with the identification of islets from the exocrine contaminants (45). Improvements in islets yields and purification of the islets from the unwanted exocrine tissue

have been attempted through a variety of means. Less viscous isoosmolar solutions such as bovine serum albumin (46, 47), dextran (48), hypaque-Ficoll (49), and percoll (50, 51) have all been attempted in place of the Ficoll. Experimental techniques such as electrophoresis (17, 36), elutriation (52), and the infusion of collagenase mixture into the medium used to disrupt the pancreas (in vitro ductal perfusion) (53) are all methods being examined to increase islets yields (52).

Currently, intralobular distension, dissociation by collagenase digestion and Ficoll purification are the standard methods of isolating pancreatic islets of Langerhans in rats, yielding 300 to 400 islets per adult rat pancreas (13, 39).

Partial amelioration of hyperglycemia, polyuria and glycosuria was achieved in 1972 by Ballinger and Lacy who transplanted 400 to 600 isolated islets into either the peritoneal cavity or intramuscularly into the thigh muscle of rats (54). The rats had been experimentally induced into a diabetic state by the injection of the B cell toxin, streptozotccin (54). Reckard et al., independently showed that repeated injections of isolated islets intraperitoneally normalized blood glucose levels (55). Kemp et al., in 1973, further improved the normalization of blood glucose levels following islet transplantation by injection of isolated islets into the liver via the portal vein (56, 57).

The specific site of islet transplantation has been shown to be critical for the utilization of the secreted insulin (13, 18). Reversal of an experimentally-induced diabetic state has been attempted in many locations, including subcutaneously (17), intratesticular (58, 59), intracerebral (60), and renal subcapsular space of the kidney (13, 61). However, the transplantation sites associated with portal drainage, such as intraportal injection to the liver (56, 62), or intrasplenic (63, 64) transplantation have been shown to be the most effective locations (13).

Experimental islet transplantation has been able to reverse experimental diabetes in many animal models (54, 63, 65) and thereby prevent the appearance of late complications of diabetes (66-69). Current advances in the ability to isolate highly purified islets from human pancreases (70-72) have recently made clinical islet transplantation a possibility (73, 74). One of the remaining obstacles to the establishment of large scale clinical islet transplantation programs is the inability to isolate sufficient islet yields required for successful transplantation. The feasibility of any islet transplantation program depends on the availability of viable islet tissue for transplantation.

The transplantation process requires that the isolated organ be preserved <u>in vitro</u> for a specific period of time.

A period of twenty-four hours is generally required for the completion of human leukocyte-associated (HLA) antigen tissue typing, recipient screening, crossmatching and for the selection of the best recipient for organ transplantation (13, 75, 76). The longer preservation time would provide more flexibility for a world-wide sharing of donated organs.

In organ preservation, the anatomical characteristics and metabolic needs of the organ, its resistance to damage from ischaemia, and its sensitivity to manipulation must be evaluated when specific preservation protocols are being established (13, 17).

Early attempts to preserve pancreatic tissue used those preservation techniques established for kidneys. These techniques appear to have been less successful because of the pancreatic tissue's greater sensitivity and vulnerability to damage during removal and preservation (77).

Preservation of the pancreas by hypothermic perfusion requires that there is complete vascularization of the organ through cannulation of the arterial blood supply. This method requires that the pancreas be anatomically intact and requires precise techniques (77, 78). Since the pancreas is a low flow organ and needs only a small amount of blood in the tissues, massive edema results if the pancreas is perfused at too high a pressure or flow rate (78). Florack et al., (1983)

preserved canine segmental pancreas allografts for 24, 48 and 72 hours and concluded that cold storage was superior and more reliable than pulsatile machine perfusion (79).

Normothermic preservation involves perfusing the pancreas without decreasing the temperature of the pancreas or the preservation solution. By maintaining the tissue at 37°C, the damage which may be inflicted during hypothermic preservation may not occur (80). However, by using this method there is a problem of supplying the necessary energy substrates, as well as oxygen, to the tissue. A major problem in normothermic pancreas preservation is the autodigestion of the pancreas due to exocrine secretions secreted by the pancreas. Researchers have had little success using normothermic preservation because of this autodigestion and the nutrient supply shortfall (80).

Hyperbaric preservation of pancreaticoduodenal grafts were first attempted by Idezuki et al., in 1968, as a means of preserving the functional viability of the pancreas for several hours, while attempting to prevent the ischemic damage commonly associated with organ preservation (81). The purpose of this method of preservation was to inhibit metabolism and prevent tissue edema; however, the excessively high oxygen partial pressure were harmful to the tissues (81, 82, 78).

Technical difficulties also made this method of preservation impractical.

Tissue culture as a method of short term pancreas preservation is not practical because pancreatic islets must be isolated prior to culture. Culturing the pancreatic islets at room temperatures (22°C) is an expensive and time-consuming method of preservation (13), but islet culture does offer some advantages following islet isolation (83). In vitro islet culture following isolation has been shown to alter the immunogenicity of the isolated islets, resulting in improved islet allograft survival (84, 85). Cultured pancreatic islets remain functionally and morphologically intact following prolonged incubation (86, 87). Tissue culturing of isolated islets is used primarily as a means of maintaining islets for metabolic studies of the islets, rather than as a practical method of long term preservation (13).

For long term islet preservation, cryopreservation is a viable and practical alternative. Cryopreserved pancreatic islets have been shown to retain their morphological integrity and physiological capacity to control hyperglycemia following indefinite low temperature preservation in liquid nitrogen at -196°C (13, 88). Control of the cooling and thawing processes, as well as the protection of intracellular ice formation, and large osmotic fluctuations through the addition

of a cryoprotectant agent, are all critical to successful recovery of islets (89). The stepwise addition of a cryoprotectant agent, such as dimethyl-sulfoxide (Me_2SO), precedes the freezing of the islets (89). Rajotte et al., in 1981, determined that slow cooling (cooling at 0.25°C per minute to -75°C) followed by immersion into liquid nitrogen and rapid thawing (200°C per minute) provide the optimal conditions for cryopreservation of rat islets (90). Recently, it has been shown in islet transplantation studies that slow cooling (0.25°C per minute) to -40°C in combination with rapid thawing (200°C per minute) is superior to slow cooling (0.25°C per minute) to -70°C (91).

Techniques in human islet isolation may not yet be capable of isolating sufficient numbers of purified islets for islet transplantation from a single donor. Therefore, cryopreservation appears to be an effective means to store or bank islets prior to multiple donor islet cell transplantation (13, 36).

Another technique, and the most simple alternative for organ preservation, is hypothermia alone, simply decreasing the temperature of the isolated organ while immersing the organ in a preservation solution. The duration of the cold storage interval has been shown to directly influence the functional success rates of islet transplantations, whether

by whole pancreas transplantation (92), dispersed pancreas fragment transplantation (93, 94) or isolated islet transplantation (95, 96). Extended hypothermic cold storage preservation has been shown to decrease the ability to recover rat islets following preservation (97). The preservation solution employed to store the whole pancreas has also been shown to affect islet yields (97) and pancreas transplantation success (98).

Collins et al., in 1969, introduced a preservation solution which was capable of storing kidneys for up to thirty hours (99). Since that time there have been many studies which have proposed "new and improved" preservation solutions (98, 100-104). However, many cold storage solutions originally developed for kidney preservation have proven to be relatively ineffective in pancreas preservation (105). Belzer and Southard recently developed a multi-organ preservation solution, the University of Wisconsin cold storage solution (UW) (105, 106). This solution has proven to be effective in short term pancreas (107, 108) and liver preservation (109) and has also extended the cold storage time of kidney preservation (110).

Although low temperature preservation reduces the metabolic need of the preserved organ under ischemia, the low temperature itself may be injurious to cell membrane structure

and function especially that of ion fluxes (111, 112), which would lead to irreversible damage of the preserved organ. It is possible that an optimal preservation temperature, which may be tissue/organ specific, could be selected to maximize hypothermic survival despite the beneficial and the deleterious effects of low temperature. The present study was undertaken to improve the short-term rat pancreas preservation. Several hypothermic temperatures (4, 7, 10, and 15°C) were evaluated to determine the effect of preservation temperature on islet yield and subsequent islet function. A series of experiments were also designed to compare the efficacy of UW cold storage solution (DuPont, Wilmington, DW) to Euro-Collins preservation solution (Travenol, Deerfoot, IL).

For future clinical consideration, freshly isolated islets were preserved by cold storage. The islet functions were then compared to those of the islets isolated after whole pancreas preservation for the same time period. The glucose perifusion assay was used to measure the functional ability of the isolated islets to secrete insulin on demand. The insulin responsiveness of the islets were quantified and compared using the mean insulin secretion rate (MISR). The overall success or failure of hypothermic rat pancreas

preservation prior to islet isolation was also assessed using the islet preservation index (IPI).

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

Male Sprague-Dawley rats (200 - 270 g body mass) were purchased and kept in hanging individual cages at room temperature (22°C) in the Biological Sciences Lab Animal Services facility and were cared for following the guidelines of the Canadian Council on Animal Care. The animals were fed Purina rat chow and water ad lib, but food was removed 24 hours prior to sacrifice of the animal.

EXPERIMENTAL PROCEDURES

Pancreatectomy Procedures

Ductal Distension

Rats were anaesthetized with a single dose (0.4 mg/g body weight) of sodium pentobarbital (Somnotol) (M.T.C. Pharmaceuticals, Mississauga, ON) injected intraperitoneally (IP). A total pancreatectomy was performed by entering the abdomen through a long ventral midline incision and the common bile duct was identified and cannulated near the hilus of the liver with Intramedic non-radiopaque PE 50 polyethylene tubing (Clay Adams, Parsippany, NJ) attached to a 26 gauge needle

(B-D, Rutherford, NJ) (39, 90).

The main pancreatic duct of the rat pancreas drains directly into the common bile duct, therefore, following ligation of the distal end of the common bile duct with 4-0 silk (Ethicon Inc., Sommerville, NJ), intralobular distension and disruption of the pancreas occurred when the pancreas was injected with 10 mL of the specific preservation solution (either Hanks', UW or Euro-Collins solutions) at the controlled temperature of either 4, 7, 10, 15°C according to experimental protocol (± 0.1°C) (39, 90) (see Appendices A, B and C for complete solution compositions).

The distended pancreas was then carefully dissected from the duodenum, using the spleen as an anchor. The excised pancreas was then placed into a small evaporating dish and immersed with 50 mL of the specific organ preservation solution. The spleen was then removed.

Intraabdominal Vascular Flush and Ductal Distension

Rats were anaesthetized and the common bile duct was cannulated following the procedures previously described for pancreas harvest using ductal distension. However, prior to ductal distension the pancreas was cooled via infusion of the specific preservation solution (either UW or Euro-Collins solution) at 4°C (± 0.5°C) via the abdominal aorta.

The intraabdominal organs were moved to the side and covered with saline soaked gauze. Forceps were used to remove the fat and connective tissue to expose the descending aorta and separate the aorta away from the inferior vena cava. Sutures (4-0 Silk) were placed around the aorta, distally and anteriorly of the exposed site. These sutures were held by surgical clamps to occlude blood flow immediately prior to cannulation of the descending aorta. Following heparinization (100 units) (Hepalean, Organon, Toronto, ON), the abdominal aorta was cannulated with Intramedic non-radiopaque PE 190 polyethylene tubing (Clay-Adams) which had been pretreated with siliconized saline solution (10 μ/mL). The cannula was then secured in position with 4-0 silk. The descending aorta was then ligated above the coeliac axis artery and below the superior mesenteric artery. The intraabdominal organs were then cold flushed (4°C) <u>in situ</u> via gravity (approximately 40 cm water pressure) until the effluent from the vena cava was clear in color (approximately 60 - 100 mL). Upon completion of the vascular perfusion, the cooled pancreas was distended via the common bile duct with 10 mL of the specific preservation solution (either UW or Euro-Collins) using the previously described procedures for ductal distension. The distended pancreas was then carefully dissected from the duodenum, using the spleen as an anchor. The excised pancreas

was then placed into a small evaporating dish containing 50 mL of the specific organ preservation solution. The spleen was then removed.

Pancreas Preservation

The isolated pancreas was subjected to hypothermic cold storage preservation extending from 0 to 48 hours (0, 6, 18, 24, 48 hours according to preservation protocols), at fixed preservation temperatures which ranged from 4 to 15°C, in either UW or Euro-Collins organ preservation solutions, in an attempt to determine the optimal preservation temperature for short term pancreas preservation. The temperature of preservation was carefully controlled and monitored at set levels according to experimental protocol using a RTE-110 or RTE-210 digital constant temperature water bath (± 0.1°C) (Neslab Instrument Inc., Newington, NH). The preservation solution temperature was measured using a Type T copper-constantan temperature thermocouple (Cole Parmer, Chicago, Il) attached to a Thermalert TH-6 digital temperature recorder (Bailey, Saddlebrook, NJ) to ensure that preservation temperature protocols were maintained.

Isolation of Islets of Langerhans

Pancreatic islets of Langerhans were isolated using the collagenase digestion method originally described by Lacy and Kostianovsky (39) as modified by Rajotte et al. (90). For the control groups, it was not necessary to redistend the pancreas prior to islet isolation. However, the preserved pancreatic tissue from the preservation groups was redistended with 12 to 15 mL of cold (4°C) supplemented Hanks' Balanced Salt Solution (HBSS) (Gibco, Grand Island, NY) which contained 100 mg/dL D-glucose (Abbott Laboratories Ltd., Montreal, PQ), 100 U/mL penicillin (Whittaker Bioproducts, Walkersville, MD) and 100 µg/mL streptomycin (Whittaker Bioproducts, Walkersville, MD). The distended tissue was then placed into a 100 x 15 mm plastic petri dish where the unwanted connecting fat particles, contaminating lymph nodes and major vessels were excised from the pancreatic tissue and discarded.

The cleaned pancreatic tissue was then cut into small pieces (2 cm) and placed into a flat bottom evaporating dish containing chilled HBSS (4°C) which was placed in a tray containing ice-water slush mixture (0 - 4°C). The pancreatic tissue was then chopped with curved scissors (Fine Science Tools Inc., Vancouver, BC) until fragments of 1-2 mm in size were obtained. Fat and exocrine fragments which rose to the

surface during chopping were poured off and discarded and fresh cold HBSS was added. Tissue fragments were then transferred into a pre-weighed 50 cc polystyrene graduated conical tube (Corning, Corning, NY). The pancreatic tissue was then centrifuged at 1500 rpm (433 gravities (g)) for 15 seconds and the supernatant solution was removed. All centrifugation steps were performed using a Beckman GL-2 centrifuge (Beckman Instrument Inc., Palo Alto, CA) with a Beckman GH-3.7 swinging bucket rotor. Tissue weight was determined and 2 mL of cold HBSS (4°C) was added for each gram of wet pancreatic tissue. As well, 15.0 mg of collagenase (Sigma Type V lot # 27f-6815) per mL of tissue (Sigma Chemical Co., St. Louis, MO) was added to the pancreatic tissue. The final collagenase concentration was 8100 units of collagenase per mg dry weight of collagenase per mL of pancreatic tissue.

In a 37°C water bath, the tissue-collagenase mixture was vigorously hand shaken until most of the large chunks of pancreas were broken down (approximately 5.0 - 5.5 minutes when using Type V Collagenase). Addition of cold HBSS (4°C) was added to cease digestion when the pancreatic tissue and collagenase mixture had become mucoid in appearance (113). Islets had been separated from the exocrine tissue by enzymatic activity and agititation of the tissue. The tissue-

collagenase mixture was centrifuged at 1500 rpm (433 g) for 10 seconds and the supernatant was removed. Approximately 15 mL of cold HBSS (4°C) was then added to the digested tissue and gently shaken to resuspend the tissue. The mixture was then centrifuged at 1500 rpm (433 g) and the supernatant was discarded. Repeated centrifugation steps were then performed, spinning at 1000 rpm (193 g), 800 rpm (123 g), 500 rpm (48.1 g) and 1500 rpm (433 g) adding 15 mL of HBSS per spin and discarding supernatant after each spin. After the last supernatant was removed, 5 mL of 25% Ficoll (400 DL (Sigma)) solution (23°C) was added and vortexed to gently resuspend the tissue in the 25% Ficoll solution. Using an automatic pipette dispenser (Drummond, Broomall, PA) and serological pipettes, (Kimble, Toledo, OH) 5 mL of 23%, 20%, and 11% (all at 23°C) Ficoll solutions were then carefully added to produce a layered Ficoll gradient. The Ficoll was added in order of decreasing concentration. Procedures of the preparation of the Ficoll solutions are found in Appendix D. The specific density of the Ficoll solutions was confirmed using a hand-held refractometer (American Optical Company, Keene, NH). The Ficoll-pancreatic tissue mixture was then centrifuged at 2000 rpm (769 g) for 20 minutes using the swinging bucket rotor in the Beckman centrifuge. This centrifugation method was used since islets are less dense than the exocrine portion and in

a discontinuous Ficoll gradient, islets would be separated from the remaining unwanted pancreatic tissue (40).

Following centrifugation, a siliconized pasteur pipette was used to gently remove the layer of tissue from between the 11% and 20% layer and the 20% and 23% layer of Ficoll solution (first and second layer interfaces). The tissue was then placed in a siliconized pyrex test tube (Fisher, Pittsburgh, PA) (15 mL capacity) containing modified Medium 199 solution (Gibco, Grand Island, NY) (23°C) which was supplemented with Earl's Salts, 100 mg/L L-Glutamine, 10% fetal calf serum (v/v) (Gibco, Grand Island, NY), 25 mM HEPES (N-2-Hydroxyethylpiperazine-N^1-2-Ethanesulfonic acid) (Gibco), penicillin (100 U/mL) (Whittaker) and streptomycin (100 µg/mL) (Whittaker) (see Appendix E for complete solution composition). The islet tissue was then centrifuged at 2000 rpm (769 g) for 20 seconds and the supernatant was discarded. Medium 199 solution was added and two spins at 1500 rpm (433 g) were performed to gradually dilute out the Ficoll solution. The isolated islets were then transferred to a plastic petri dish (Fisher) containing supplemented Medium 199 solution.

Islet Identification

Pancreatic islets of Langerhans were identified under a binocular dissecting microscope (25 x power) (Olympus, Tokyo, Japan) with illumination from below through a # 58 green filter (Eastman Kodak, Rochester, NY) and horizontal illumination from a dual arm brilliant white fiber optic light source (Cole Parmer, Chicago, IL). Using the illumination methods described, islets appeared semi-opaque orange-red structures, round to ovoid in shape, with relatively smooth and well-defined borders (113, 114). The exocrine tissue appeared more transparent in color with irregular boundaries and were clumped together. Islets vary in size from 60 μm to 350 μm (114). Those islets within the 80 μm to 200 μm range as measured using an ocular optical micrometer grid (Wild-Leitz, Willowdale, ON) were hand-picked free of exocrine contaminants using a siliconized micro-pipette. Islets were collected and placed in siliconized vials containing supplemented Medium 199 solution for viability evaluation. The total number of islets isolated and collected after each experiment was recorded.

Isolated Islet Preservation

Islets of Langerhans were isolated using the procedure previously described. The islets were collected and placed in a 15 mL siliconized pyrex test tube (Fisher) containing modified Medium 199 solution (23°C) and centrifuged at 1500 rpm (433 g) for 15 seconds. The Medium 199 solution was removed and replaced with cold (7°C) UW preservation solution. The test tube containing the isolated islets was immersed in UW preservation solution and placed in a RTE-110 digital constant temperature waterbath which maintained the preservation temperature at 7°C (± 0.1°C) for the entire 24 hour preservation interval. Following hypothermic isolated islet preservation, Medium 199 solution (23°C) was added and two spins at 1500 rpm (433 g) were performed to gradually dilute out the UW preservation solution. The islets were then transferred to a plastic petri dish (100 x 15 mm) containing supplemented Medium 199 solution. Islets being within 80 to 200 μm in size were counted and collected and placed in siliconized vial containing modified Medium 199 solution for subsequent perifusion analysis.

VIABILITY

Histology

The morphology of freshly isolated and hypothermically preserved pancreas was examined after selectively staining the isolated islets with dithizone (diphenylthiocarbazone) (Sigma) solution, a histological stain which specifically binds to the zinc particles found within the islet mass (91, 115, 116). Freshly made stock of dithizone solution was prepared from 10 mg dithizone and 3 mL of 960 µl/mL ethanol, and 3 drops of 30% ammonium hydroxide added to the solution during mixing (115). Islets were immersed in the stock dithizone solution for 10 minutes, after which an aliquot of the islet mixture was placed on a slide and examined under the dissecting microscope (25 x power). Photographs of the isolated islets from each of the preservation groups were taken for comparative purposes.

The post-Ficoll exocrine tissue pellet was also examined using dithizone staining to verify any changes in sedimentation characteristics in the islets following hypothermic preservation. Dithizone solution (5 mL) was added to the post-Ficoll tissue pellet following islet isolation. The mixture was gently vortexed and left to settle for 10-15

minutes. Aliquots were removed and examined under the dissecting microscope for red-stained islets embedded or still attached to partially digested exocrine tissue. Photographic plates of fresh and preserved post-Ficoll exocrine tissue pellets demonstrated the differences between the groups.

Glucose Perifusion

Viability of the isolated islets of Langerhans was assessed using glucose perifusion. The glucose perifusion technique, a functional metabolic test, was used to evaluate the rate of insulin secretion from a known number of specific-sized isolated islets when challenged with varying concentrations of glucose. The procedures used for the perifusion followed Lacy et al. (117).

Perifusion Solutions

RPMI 1640 solution (Gibco) which had been supplemented with 10% Fetal calf serum v/v (Gibco), penicillin (100 U/mL), streptomycin (100 µg/mL), 2 grams of sodium bicarbonate (Fisher) and 1.9 mL of 5 N NaOH (Fisher) was used as the stock perifusion solution (see Appendix F for complete solution composition). The two glucose solutions, 50 mg/dL

and 500 mg/dL, were made from the stock RPMI solution with the addition of 50% dextrose solution (Abbott). The solutions were placed in 500 mL Erlenmyer flasks and set in a 37°C waterbath. The perfusate was oxygenated by retrograde flow of a mixture of 95% O_2 and 5% CO_2 (Carbogen) (Union Carbide, Toronto, ON) introduced by polyethylene tubing at the bottom of each of the flasks.

Perifusion Chambers

The perifusion chambers used in the perifusion were made from modified Micro-syringe filter holders (Millipore, Bedford, MA) with a Perfektum stainless steel 16 gauge hypodermic needle (Popper and Sons, New Hyde Park, NY) drilled and soldered into the top of the filter holder at a 45 degree angle. A three-way stopcock with male Leur lock (Cobe, Lakewood, CO) was attached to the needle. A plastic tubing adapter (Clay Adams, Parsippany, NJ) with a brass fitting attached to the leur lock portion of the top of the perifusion chamber enabled the system to become air tight. Inside the perifusion chamber there was a pretreated 25 μm thick nitex mesh filter (BSH Thompson, Toronto, ON), a 3/16" width teflon O-ring, stainless support screen and bottom teflon gasket.

Perifusion Circuit

The perifusion solution was passed through a proportioning peristaltic pump (Technicon, Terrytown, NY) with grey-grey manifold pump tubing (0.051 I.D.) (Fisher) providing a flow rate of 1 mL/minute to the perifusion chambers which were immersed in 37°C water jacketed beakers (1 L capacity) (Cole Parmer). The effluent from the chambers passed via polyethylene sampling lines (PE 160 tubing) (Clay-Adams) to a modified fraction collector (Model 201) (Gilson, Villiers Le Bel, France). The circuit of tubing for the perifusion system consisted of I.V. tubing (Cutter Biological, Etobicoke, ON) attached to the chambers via plastic tubing adapters (Clay Adams). The temperature of the perifusion circuit was maintained at 37°C through the use of a RTE-110 or RTE-210 digital constant temperature water bath (± 0.1°C) (Neslab Instrument Inc., Newington, NH) as monitored using a Type T copper-constantan temperature thermocouple (Cole Parmer, Chicago, Il) attached to a Thermalert TH-6 digital temperature recorder (Bailey, Saddlebrook, NJ).

Loading of Islets

Batches of a known number of isolated islets of Langerhans of specific sizes (80 μm to 200 μm range) were

transferred to the perifusion chambers via a barrel from a 10 mL syringe attached to a three-way stopcock attached to the needle at the top of the perifusion chamber. Attempts were made not to allow any air into the system during the islet loading. Once all islets were loaded, the programmed sequence on the fraction collector samples was initiated and effluent was collected.

Perifusion Sampling

Islets loaded in the perifusion chambers were perfused with modified RPMI 1640 solution containing glucose in the following concentrations: initially 50 mg/dL glucose for 60 minutes, then 500 mg/dL glucose for the next 60 minutes. For the last hour of the perifusion, islets were returned to the 50 mg/dL glucose solution. The effluent from the perifusion chambers was collected (2 mL sample) at 10 minute intervals with additional samples taken at 1, 3, 5, 7, and 9 minutes after the switch to the high glucose stimulatory phase of the perifusion (118). The perifusion sampling sequence is found in Appendix G. A total of 20 samples were collected throughout the perifusion. The samples were collected in an ice bath container and stored at -17°C until the insulin content could be measured.

Insulin Determination

Insulin content from the perifusion samples were determined using insulin double antibody radioimmunoassay RIA kits (Pharmacia, Uppsala, Sweden) according to the methods originally developed by Morgan and Lazarow (119). Standard working solutions were made using rat insulin (Lot # 850918) (Novo Biolabs, Bagsvaerd, Denmark) and Pharmacia Diluent solution (Pharmacia). The principle behind radioimmunoassays is that insulin in the perifusion sample competes with insulin-I^{125} for specific binding sites on the guinea pig antibody. The free insulin and the bound insulin on the antibody are separated by centrifugation and decanted following the addition of the second immunoabsorbant. The radioactivity in the remaining pellet is inversely proportional to the quantity of insulin in the sample.

The insulin samples were run in duplicate following the procedures outlined in the RIA kits. A 100 μL of each standard insulin solution or the 100 μL of unknown sample was added to 50 μL of Insulin-I^{125} (approximately 37 kBq) and 50 μL of antibody (antiserum raised in a guinea pig) in 12 x 75 mm glass culture tubes (Fisher). The rat insulin solution standards used to generate the standard curve were 0, 3, 6, 15, 30, 45, 60, 90, and 180 μ U insulin/mL. Samples were

diluted and redone if their calculated radioactivity values were out of the range of the standard curve.

Contents of the test tubes were gently mixed and then incubated at room temperature (22°C) overnight. Following the incubation period, 2.0 mL of decanting suspension (Sepharose anti-guinea pig, immunoglobulin G) was added and the tubes were further incubated for 30 minutes at room temperature (22°C). The mixture was then centrifuged (1000 X g for 15 minutes) and then decanted for approximately 2 minutes onto absorbent paper. The radioactivity of the test tubes was determined using a 4/600 Micromedic Automatic Gamma counter (Rohn and Hass, Horsham, PA) with a counting time of 2 minutes. Radioactivity counts were expressed as counts per million (cpm) and percentage binding of the unknown samples was calculated as a percentage of the count of 0-standard using a Model 43 Micromedic Assay Compucenter (MACC) (Rohn and Hass). The rat insulin standard curve was calculated and the insulin values were then extrapolated from the standard curve. Insulin values are expressed as μ U of insulin.

Perifusion graphs, plotting the insulin secretion rate per islet over the time course of the perifusion, were then generated using a database and graph generating program.

STATISTICAL ANALYSIS

Islet yield results are expressed as mean number of islets per experiment (2 pancreases) ± standard error of the mean. For comparison, mean islet yields from each of the preservation grou were analyzed using a standard unpaired t-test using the SPSS-PC statistical package. Differences between the preservation groups and the non-preserved controls were also compared with one-way analysis of variance and subsequent Student Newman-Keuls test using the SPSS-PC statistical package. MISR values collected during glucose perifusion were analyzed using the unpaired t-test and one-way analysis of variance. IPI values were compared using one way analysis of variance. In all cases, significant differences were assumed if the probability (p) was less than 0.05.

RESULTS

ISLET YIELD

A. Control:

Ductal Distension

The effect of various preservation solutions on islet yields using ductal distension via the common bile ducts are shown in Figure 1. If Hanks' balanced salt solution (HBSS) was used to distend the pancreas, islet yields were 974.1 ± 73.1 (sem) (n = 10) islets per experiment (2 pancreases). However, if UW solution was used to distend the pancreas prior to islet isolation, islet yields were reduced to 780.3 ± 21.8 (n = 7) islets per experiment. Islet yields were further decreased if Euro-Collins solution was used to distend the pancreas, resulting in only 468.6 ± 15.1 (n = 7) islets per experiment. Islet yields from distension via HBSS and UW solutions were significantly higher than the Euro-Collins solution group ($p < 0.0001$). There were also significant differences between the pancreases that had either been distended with UW or HBSS solution ($p = 0.038$).

Vascular Flush and Ductal Distension

Intraabdominal vascular flushing prior to islet isolation resulted in a significant decrease in islet yields, using both UW and Euro-Collins solutions (Figure 2). If UW solution was used to flush the pancreas, an average of 497.6 ± 26.0 (n = 7) islets were recovered per experiment. A further decrease in islet yields resulted if Euro-Collins solution was used yielding an average of only 295.4 ± 19.3 (n = 7) islets per experiment. Islet yields following vascular flush using either UW or Euro-Collins solutions were significantly different from each other ($p < 0.0001$).

A comparison of the two control groups, ductal distension and vascular flush with subsequent ductal distension, as shown in Figures 1 and 2, indicate that ductal distension is significantly superior to vascular flush and subsequent ductal distension ($p < 0.0001$) and that UW preservation solution is statistically significantly superior to Euro-Collins preservation solution ($p < 0.0001$) in pre-treating the pancreas for subsequent isolation of the islets of Langerhans.

B. Comparison of UW and Euro-Collins Preservation Solutions:

Hypothermic preservation for 24 hours at 4°C using UW organ preservation solution following ductal distension via the common bile duct was statistically superior ($p < 0.001$) to Euro-Collins preservation solution, resulting in 318.0 ± 10.1 islets (per 2 pancreases) ($n = 7$) from the UW solution group, and fewer than 10 islets from the Euro-Collins solution group ($n = 7$) (Figure 3).

Islet yields from pancreases that had been hypothermically preserved for 24 hours at 4°C following intraabdominal vascular flushing and subsequent ductal distension prior to pancreas preservation, from both preservation solutions groups, decreased drastically ($p < 0.0001$) yielding fewer than 10 islets per experiment (Figure 3) ($n = 7$).

Significantly superior results were found using ductal distension ($p < 0.0001$) and hypothermic preservation using UW solution ($p < 0.0001$) than using vascular flush and ductal distension together and using Euro-Collins solution. Therefore, all subsequent preservation experiments were performed using ductal distension via the common bile duct and hypothermic cold storage in UW preservation solution.

C. Effects of Duration of Hypothermic Preservation:

As seen in Figure 4, there is a progressive decrease in the ability to recover islets with prolongation of the hypothermic preservation at 4°C. Islet yields diminished significantly from control values ($p < 0.0001$) to 464.4 (± 26.5) after only 6 hours of hypothermic preservation following ductal distension and cold storage in UW preservation solution; further reductions in islet recovery to 383.0 (± 11.5) occurred after 18 hours of cold storage; and to 318.0 (± 10.1) following 24 hours cold storage. Less than 10 islets were recovered after 48 hours of cold storage in UW solution at 4°C ($n = 7$ for each group in this series of experiments). These results indicate that there is a time-dependent deterioration in the ability to isolate islets following hypothermic preservation at 4°C.

D. Effects of Temperature on Hypothermic Preservation:

After ductal distension with UW preservation solution and hypothermic preservation for 24 hours at varying hypothermic temperatures (Figure 5), the mean islet yield was highest at 10°C (466.7 ± 28.5) compared to 7°C (410.9 ± 24.4), 4°C (318.0 ± 10.1), and 15°C (44.6 ± 3.9) ($n = 7$ for each

preservation group). At any given temperature, 24 hour cold ischemia resulted in significantly fewer islets recovered compared to control values ($p < 0.0001$). The islet yield from the 4°C preservation group was significantly different from both the 7°C ($p = 0.012$) and the 10°C group ($p < 0.002$). While there was no significant difference between the 7 and 10°C preservation groups ($p = 0.194$), the 4, 7 and 10°C preservation groups were significantly different from the 15°C group ($p < 0.0001$).

Extending the preservation interval to 48 hours resulted in drastic reductions of mean islet yields for all preservation groups (4°C, 6.1 ± 1.0; 7°C, 6.9 ± 1.2; 10°C, 5.3 ± 2.3; and 15°C, 2.9 ± 0.5) ($n = 5$ for all groups of 48 hour preservation experiments).

These experiments indicate that the temperature of hypothermic preservation is critical to the subsequent successful recovery of islets of Langerhans following a 24 hour preservation interval, and that preservation at 7 and 10°C is superior to cold storage at 4°C.

ISLET VIABILITY

The ability of isolated islets of Langerhans to secrete insulin when challenged by various concentrations of glucose solutions was used as a quantitative functional test to evaluate the impact of the temperature used and the duration of pancreas preservation.

A. Control:

The glucose-stimulated insulin response of the freshly isolated islets from the control group produced a biphasic pattern of insulin release (Figure 6). Following an initial baseline secretion of 0.073 μ U/islet/min in the low glucose solution (50 mg/dL), an approximately 12-fold increase in insulin secretion was observed when the islets were subjected to high glucose solution (500 mg/dL). The peak of the first phase, 0.935 μ U/islet/min, occurred approximately three minutes following initiation of the high glucose stimulation. The second sustained phase of glucose-induced insulin secretion followed with the peak of 1.478 μ U/islet/min. Insulin secretion returned to and remained at near pre-stimulation levels within 20 minutes following the conclusion of the high glucose challenge phase of the glucose perifusion.

B. Hypothermic Pancreas Preservation:

Islets of Langerhans, isolated following 24 hour hypothermic preservation in UW organ preservation solution from all preservation groups exhibited a slight biphasic pattern of insulin secretion; however, the quantitative amount of insulin released was significantly reduced ($p < 0.0001$) from control level in all groups following hypothermic preservation (Figure 7).

The mean initial baseline insulin secretion levels (μ U/islet/min) were 0.179 for the islets isolated from pancreatic tissue which had been stored for 24 hours at 4°C, 0.127 for the 7°C group, and 0.154 for the 10°C preservation group (Figure 7). Islets from the 7°C preservation group displayed the highest response during glucose stimulation followed by that of the 10°C and then the 4°C group. The glucose-stimulated increase in insulin secretion was approximately 5-fold at 7°C, 3.3-fold at 10°C and 1.4-fold at 4°C for the first peak and a 7.2, 4.7, and 2.9-fold increase, respectively, for the second peak of the glucose stimulated insulin secretion (Figure 7).

A delay in the return of the islets to baseline levels following termination of the glucose challenge was noted in all preservation groups; the 4°C group appeared to be the

slowest to return to baseline (Figure 7) and the 7°C group showed the quickest return to near pre-stimulation levels. The return to baseline insulin secretion levels was significantly slower in all preservation groups as compared to the insulin secretion levels found in the islets from the control group. Insulin secretion levels remained relatively constant for the remainder of the perifusion with a progressive decrease in insulin secretion. There were no perifusions were performed on islets collected following 24 hour preservation at 15 °C and 48 hour preservation at all temperatures due to insufficient islet yields.

An index was developed to quantify the secretory pattern of insulin release during the entire glucose perifusion. This index, the mean insulin secretion rate (MISR) is calculated by dividing the insulin values from the high glucose stimulatory phase by both periods of low glucose stimulation. Further clarification on calculation of the MISR index is found in Table 1 and graphically in Figure 8. As presented in Table 2, the MISR, was highest for the 7°C preservation group (2.73) but was only 54.9% of the control value of 4.97. The 10°C group had an MISR value of 2.42, 48.7% of the control and the 4°C group had the lowest MISR, 1.77, only 35.6% of the control value. All preservation groups were statistically significant from the freshly isolated islets ($p < 0.0001$).

MISR values at 4°C differed significantly ($p < 0.0001$) from both 7 and 10°C groups, as did MISR values at 7°C from those at 10°C ($p = 0.031$). The total islet yields in both the control group and the experimental groups and the islets' ability to secrete insulin were considered in a combined index, the islet preservation index (IPI). Using the IPI, a second index developed herein, a further separation of the 4°C preservation group from the 7 and 10°C groups was evident. The IPI values were 0.722, 1.44, and 1.45 for the 4, 7 and 10°C preservation groups respectively (Table 2). There were no significant differences in IPI values between the 4°C and the 7 and 10°C groups ($p < 0.0001$). There were no significant differences in IPI values between the 7 and 10°C groups ($p = 0.91$; one-way analysis of variance).

E. Hypothermic Preservation of Isolated Islets:

Freshly isolated pancreatic islets were stored in UW preservation solution at 7°C for 24 hours prior to glucose perifusion (n = 24 perifusion chambers from 12 animals) (Figure 9). Following an initial baseline secretion of 0.021 μ U/islet/min in the low glucose solution (50 mg/dL), an approximately 17-fold increase in insulin secretion was observed when the islets were subjected to high glucose

solution (500 mg/dL). The peak of the first phase of the insulin secretion, 0.359 μ U/islet/min, occurred approximately three minutes following initiation of the high glucose stimulation phase of the perifusion. The second sustained phase in the high glucose induced insulin secretion followed with a peak occurring at 0.554 μ U/islet/min. A blunted secretory response in the high glucose stimulated insulin release, similar to the 24 hour preservation groups, occurred in the 7°C islet preservation group. Pre- and post-glucose stimulation values appear to be lower in the islet preservation group than those found in the hypothermic pancreas preservation group. The return to basal insulin secretion levels at the termination of the glucose stimulation was much quicker in the islet preservation group (Figure 9). These results indicate that the preserved isolated islets show a superior ability to control insulin secretion as compared to islets from hypothermically preserved pancreases. These observations become further evident when the MISR index was evaluated. The MISR value for the preserved islet group was 4.91 ± 0.181, almost 99% of the 4.97 control value ($p = 0.859$, unpaired t-test). The MISR value (4.91) from the islet preservation group was significantly greater than the MISR value (2.73) from the 7°C pancreas preservation group ($p < 0.0001$).

HISTOLOGY

H. Islet Identification:

Positive identification of pancreatic islets of Langerhans was confirmed following dithizone incubation and examination of the pink-red stained islets under the dissecting microscope. Photographic plates illustrating the ability of dithizone to selectively stain the freshly isolated islets and islets isolated from hypothermically preserved pancreases are presented in Plates I and II respectfully.

I. Examination of the Post-Ficoll Exocrine Tissue Pellet:

Dithizone staining of the post-Ficoll exocrine pancreatic tissue pellet revealed that there were islets still attached to undigested exocrine tissue in all preservation groups (Plates III-IV). Islets attached to clumped masses of undigested exocrine tissue were also found in freshly isolated post-Ficoll exocrine pellets (control group) (Plate V). However, the actual number of whole and fragmented islets found in fresh controls were less than those found in the preservation groups. The "trapped" islets in the post-Ficoll exocrine pellet may be a result of inadequate distension of

the tissue following hypothermic preservation (94, 95). The intralobular distension of pancreatic tissue needed for the physical separation of the endocrine portion from the exocrine portion and the exposure of the collagenase enzyme to the pancreatic tissue may not occur if the pancreas is not sufficiently redistended following preservation.

J. Examination of the Ficoll Gradient Purity:

A noticeable change in the purity of the upper Ficoll layers during islet isolation was noted in all hypothermic preservation groups. Photographic Plate VI illustrates the typical green gel technique of observing freshly isolated "clean" islets found in the first and second layer interfaces following Ficoll purification. Photographic Plate VII displays the contamination of exocrine tissue into the first and second layer interfaces following Ficoll separation in pancreases that have been hypothermically preserved. This decrease in purity of the Ficoll layers in islet isolation following hypothermic preservation may be partially explained by cellular swelling of the exocrine tissue during hypothermic preservation. The accumulation of cellular water may alter the density of the exocrine tissue preventing successful separation of the exocrine tissue from the endocrine tissue.

As a result of the "dirty" islet collections following Ficoll purification in all hypothermic preservation groups, islets were double picked if subsequent perifusion studies were to be done. No further attempts to remove or alter the exocrine tissue and thus prevent the exocrine tissue from appearing in the Ficoll layers were undertaken in this study.

DISCUSSION

Recent improvements in experimental islet research, specifically in the mass isolation of islets of Langerhans, have lead to the first successful clinical islet transplantation (73, 74). With further advances in islet research, clinical islet transplantation may soon become a common surgical procedure. To expedite this ambition, the optimal conditions necessary for the successful isolation of islets following the obligatory period of organ storage must be evaluated. Work in this study has focused primarily on determining the optimal conditions for short term hypothermic rat pancreas preservation. The actual temperature of whole pancreas preservation prior to the isolation of the islets of Langerhans was evaluated with the aim of improving or maintaining the ability to isolate islets following the period of organ storage.

The first series of experiments dealt with the methodology of pancreas removal prior to whole pancreas storage. The results have shown that pre-storage ductal distension via the common bile duct is superior to pre-storage vascular flushing of the pancreas via the descending aorta prior to islet isolation. The diminished islet yields and subsequent poor function as a result of vascular flush has

recently been theorized to be due to inadequate flushing and consequently prolonged organ ischemia during organ harvesting (94, 95). The failure to recover islets following aortic flushing may also be a consequence of the excessive pressure used during flushing (13). Interstitial edema caused by *in situ* vascular perfusion may lead to an increase the internal osmotic pressures within the cell, leading to ionic imbalances and cell lysing (13). The dramatic reduction in islet yields following vascular flushing has recently been shown to occur in other pancreas preservation studies (94, 95). Munn *et al.*, (1989) have recently shown that, in islet yield and subsequent islet transplantation experiments, in both rat and canine models, that the vascular flush method was inferior to ductal distension of the pancreas (95, 96) Kneteman *et al.*, (1990) found poor islet function from human pancreases that had been *in situ* cooled with UW solution and stored for periods longer than 18 hours (120). All subsequent experiments in this study were therefore performed using ductal distension based on these findings.

An experiment was designed to evaluate a recently developed synthetic preservation solution, University of Wisconsin (UW) cold storage solution, and compare it to the commonly used Euro-Collins organ preservation solution for the best efficacy in whole rat pancreas preservation.

Results from this study indicate that UW solution is superior to Euro-Collins solution in both ductal distension and vascular flush experiments. Islet yields were significantly reduced following a 24 hour preservation bout (Figure 3). However, the reduction was not as severe in the experimental group that had been preserved in UW preservation solution. The components contained within UW solution (see Appendix B) apparently provided better protection of the essential cellular functions, prevented cellular swelling, and supplied the necessary nutrients needed for the successful isolation of islets following cold storage. Based on these finding, all subsequent experiments were performed using the UW organ preservation solution.

The temperature at which the pancreas was immersed for the preservation interval was evaluated to see if the actual temperature of hypothermic preservation had any effect on islet recovery and subsequent islet function. Many current preservation techniques utilize deep hypothermia (0 - 5°C) as a means of minimizing cellular damage and of decreasing the ongoing metabolic processes (106). Moderate hypothermia (7 - 10°C) increases the cell's metabolic needs more than deep hypothermia. Consequently, replenishment of the energy stores of the tissue by increasing the metabolic nutrient supply in the preservation solution is required to support the higher

metabolic activity at 7 to 10°C. Rationale for using hypothermic conditions for organ preservation is based on the principle that ongoing metabolic processes within the cell are temperature-dependent processes (121, 122). By decreasing the organ's temperature, the "number of activated molecules available to take part in physiological processes are reduced and the metabolic rate is slowed down," potentially increasing the storage time by reducing the demand for cellular metabolites (122). Oxygen consumption, a measure of metabolism and a critical metabolic factor in energy production, is 3% of the normal value at 0°C (106, 123). Belzer and Southard (1988) have calculated that cooling an isolated organ from 37°C to 0°C should extend the preservation time by a factor of 12 to 13 based on a Q_{10} of 2.0 (106).

The obvious benefits of extended hypothermic preservation may be counteracted, however, by the underlying deleterious effects of low temperature itself. Low temperature could induce alterations of the lipid portion of the cellular membrane leading to increases in membrane viscosity and eventually a separation of the lipid and integral membrane proteins if the critical temperature (T_c) for liquid crystalline to gel phase transition is reached (111, 124). Cooling of tissues during hypothermic preservation has also been shown to reduce the activity of the essential membrane

ion exchange mechanisms, specifically the Na^+-K^+ ATPase system (112, 125). The loss of ionic regulation has been shown to result in an intracellular gain of extracellular sodium leading to an increases in cell water content, cell swelling, deterioration of cellular function and eventually cell death (112, 126). The inability to regulate the intracellular calcium concentration at low temperatures, leading to the accumulation of intracellular calcium, may also be involved in the deteriorating of cells in isolated organs (111, 127). An excessive calcium accumulation inside the cell may be due to a reduced capacity for calcium efflux as the hypothermic conditions cause a decrease in the capacity to pump calcium out of the cell leading to the deterioration of the cell's ionic gradients (121, 127). By attempting to keep the calcium concentration very low in the preservation solutions, mimicking intracellular cation concentrations to extracellular concentrations and temporarily removing the transmembrane ionic imbalances, cell energy may be conserved (106). Also, by maintaining the organs at preservation temperatures nearer to or above the critical temperatures where these damaging effects arise, improvements in hypothermic preservation of organs should, theoretically, result.

There have been few studies which have specifically addressed the subject of the optimal temperature for short

term organ preservation. Work by Belzer has shown that the viability of cultured kidney tubule cells was highest at 6°C (128). Kruuv et al., studied the cell size as a function of preservation temperature and reported that for cultured lung fibroblasts storage, the optimal storage temperature is at 10°C (129, 130). Winchell and Halasz (1987) have shown that creatinine clearance was significantly improved if the rabbit's kidney was perfusion-preserved at 8°C rather than at 0°C (131). Recently, it has been shown that mild hypothermic (23°C) perfusion preservation results in better functional preservation of kidneys than either cold (4°C) or normothermic (37°C) preservation (132). Using a lung perfusion model, preservation at 10°C was found to be superior to preservation at 15°C and 4°C (133). Continuous liver perfusion at 10°C for 24 hours showed significantly higher tissue adenine nucleotide levels than those continuously perfused at 4°C (134). This study also revealed that cold storage of rat liver tissue at 10°C in Marshall's preservation solution was superior to simple cold storage at 4°C (134).

The apparent success of these preservation studies at slightly higher temperatures in other organ systems stimulated the present study. Results from this study have indicated that islet yields were significantly higher in pancreases that were hypothermically preserved in UW solution at 7 and 10°C as

compared to 4°C. However, following 24 hours of hypothermic pancreas preservation at any temperature resulted in significantly fewer islets that were harvestable than from the control group. This suggests that islet fragility and disintegration increase following in vitro hypothermic preservation within the temperature range studied. Notably, the loss of recoverable islets was most significant at 15°C, followed by that at 4°C and then 7°C and 10°C (Figure 5). The ability to isolate islets following preservation is essential to the success of future islet transplantation studies. Our results have shown that preservation at intermediate low hypothermic temperatures (7 - 10°C) can result in a greater islet harvest.

Following islet isolation, it is critical that the viability of the islets be verified. The ability and the kinetics of the isolated islets to secrete insulin following glucose challenge was chosen as the bioassay. Our results (Figure 7) indicated that islets from all preservation groups retain a limited ability to secrete insulin following high glucose stimulation (a slight biphasic pattern), although their levels of insulin secretion were significantly lower than those from the freshly isolated controls. These perifusion results compliment recent canine islet transplantation studies which have shown a time-dependent

deterioration of pancreas function following prolonged hypothermic preservation (94, 135). Islets from the 7°C preservation group demonstrated a higher ability to secrete insulin as compared to islets collected from the other preservation temperatures. An increased delay in the islets' insulin response to return to baseline following hypothermic preservation indicates a deterioration of the islets' ability to precisely recognize the varying concentrations of the glucose stimulation and to "turn off" the insulin secretion accordingly. The initial basal levels of insulin during the perifusion have also been used as an indication of islet damage (136). All preservation groups showed a higher basal insulin level than the control group, with the highest initial insulin level found in the 4°C group. A period of tissue culture following islet isolation has been shown to allow sufficient metabolic recovery of the islets and restore insulin secretion capabilities to near control levels (136). However, this study focused primarily on islet yields and insulin secretion capabilities immediately following islet isolation.

We have developed a ratio, the Mean Insulin Secretion Rate (MISR) (Table 1 and Figure 8) to quantify the insulin response to glucose stimulation in the perifused islets. The MISR is a modification of the perifusion stimulatory

factor of Foreman and Taylor (1989) developed for viability assessment following cryopreservation of islets (136). Foreman and Taylor compared the insulin secretion during the initial non-stimulatory period to the insulin secreted during the stimulatory period of the glucose perifusion, whereas our ratio provides the islets' response throughout the entire perifusion, before and after the high glucose stimulation (Figure 8). This is functionally more appropriate since there is a delayed return of insulin secretion to baseline following the termination of high glucose stimulation. A low MISR value would, therefore, reflect the damage and impairment of islet function following preservation and islet isolation. A high basal insulin secretion, and a diminished stimulatory response, including a delay in the islets' response following high glucose stimulation to "turn off" the insulin secretion, are likely to result in low MISR values. The MISR values of all preservation groups studied were significantly lower than that of the control group (Table 2). The lowest MISR was from 4°C (1.77) and the highest MISR was from 7°C (2.73); the 10°C group (2.42) was intermediate (Table 2).

The Islet Preservation Index (IPI), a new parameter developed in this study, combines both the mean islet yield before and after preservation and the insulin response (MISR) during glucose perifusion. The IPI thus reflects accurately

a measure of the overall success in preservation. If, for example, a preservation group had a low islet yield but had high insulin secretion capabilities, the IPI would have taken such disparity into consideration. The IPI values for the 7°C and 10°C preservation groups were very similar ($p = 0.90$) and were more than double that of the 4°C preservation group ($p < 0.0001$) (Table 2). We have found that even though 7°C preservation resulted in fewer islets being recovered, those islets had a higher secretion capability than islets isolated following 24 hours storage at 10°C. Both groups were more successful than preservation at 4°C. The overall superiority of preserving pancreatic tissue at these slightly higher preservation temperatures is convincingly evident. This work constitutes a significant step towards the development of a successful large scale clinical islet transplantation program.

An experiment comparing the function of isolated islets stored in UW solution at 7°C for 24 hours and islets isolated following 24 hour whole pancreas cold storage in UW solution at 7°C was undertaken to determine if there were any functional differences in whole pancreas preservation as opposed to isolated islet preservation. Results have shown a significant difference between the two groups (Table 3). The quantitative amount of insulin secreted during the stimulatory phase of the glucose perifusion was significantly

lower in the preserved islet group than in the whole pancreas preserved group (Figure 9). By preserving the islets as opposed to the whole pancreas, islets appear to retain the ability to "turn on" and "turn off" insulin secretion capabilities as seen in the control group (Figure 6). It is not clear if this is due to less islet deterioration or to a metabolic recovery during the preservation bout. The period of hypothermic preservation may be sufficient to allow some metabolic recovery following the very damaging effects of the islet isolation processes. A period of cold islet preservation may also serve to dilute out or remove any residual insulin molecules found in the outer membrane of the islet mass which may have leaked or been damaged during isolation processes. Islets which have been perifused immediately after islet isolation, may be damaged and have insulin molecules leaking out of the membrane which normally would not be expelled out of the islet. A period of islet culture, even cold islet preservation at 7°C, appears to be beneficial to islet recovery. The MISR value from the isolated islet preserved groups was significantly higher than the whole pancreas preserved group (4.91 vs 2.73). However, the MISR value from the islet preserved group showed no difference than from the freshly isolated control group even though the insulin secreted during the stimulatory phase was

approximately three fold higher in the control group (Table 3). The pre-glucose stimulation insulin values were lower and the return to low insulin secretion occurred faster in the islet preservation group than in the control group. These observations have lead to the argument that even at 7°C, there is some metabolic recovery of the islet function. Short term preservation via preservation of isolated islets is an effective alternative to whole pancreas preservation.

CONCLUSION

Our experiments in this study have demonstrated that:

1. University of Wisconsin organ preservation solution (UW) is superior to Euro-Collins preservation solution in 24 hour hypothermic *in vitro* whole rat pancreas preservation prior to islet isolation.

2. Ductal distension via the common bile duct is superior to vascular flushing the pancreas prior to islet isolation both in fresh and in 24 hour hypothermically whole rat pancreas preservation.

3. The temperature used in hypothermic pancreas is critical to the successful recovery of islets following 24 hour *in vitro* hypothermic storage; pancreases preserved at 7 and 10°C for 24 hours yield significantly higher numbers of islets than pancreases preserved at 4°C.

4. Prolongation of the preservation period at any preservation temperature leads to a progressive and significant loss of recoverable viable islets.

5. Isolated islets following 24 hour pancreas preservation show a significant reduction in insulin secretion capabilities (64.4% for 4°C, 51.3% for 10°C, and 45.1% for 7°C) following glucose challenge as compared to that of islets from the freshly isolated pancreases.

6. Islets which have been isolated from fresh pancreases and preserved at 7°C in UW preservation solution for 24 hours show a reduction in the insulin response similar to that from islets isolated after 24 hour whole pancreas preservation, however, the former display a significantly better regulation of insulin secretion prior to and following the high glucose challenge than the latter.

7. Of the preservation temperatures evaluated, whole pancreases preserved in UW solution at 10°C and 7°C were superior to 4°C based on the product of mean islet yield and the total insulin output following glucose challenge.

8. Hypothermic preservation of the pancreas above the standard temperature of 4°C should be evaluated in other animal models and eventually in humans to further improve islet harvest for subsequent islet transplantation.

FIGURE 1:

Islet isolation immediately following ductal distension via the common bile duct with Hanks' solution yielded the highest number of islets (974 ± 73.2 islets per experiment (2 pancreases)). Islet yields were reduced in the UW solution group (780.3 ± 21.8) and further reduced in the Euro-Collins solution group (468.6 ± 15.1). Bars represent +1 standard error of the mean. N = 10 (20 animals) for the Hanks' control group and n = 7 (14 animals) each for the UW and Euro-Collins' control groups. Bars sharing identical symbols indicate significant difference ($p < 0.05$) in mean islet yields between groups.

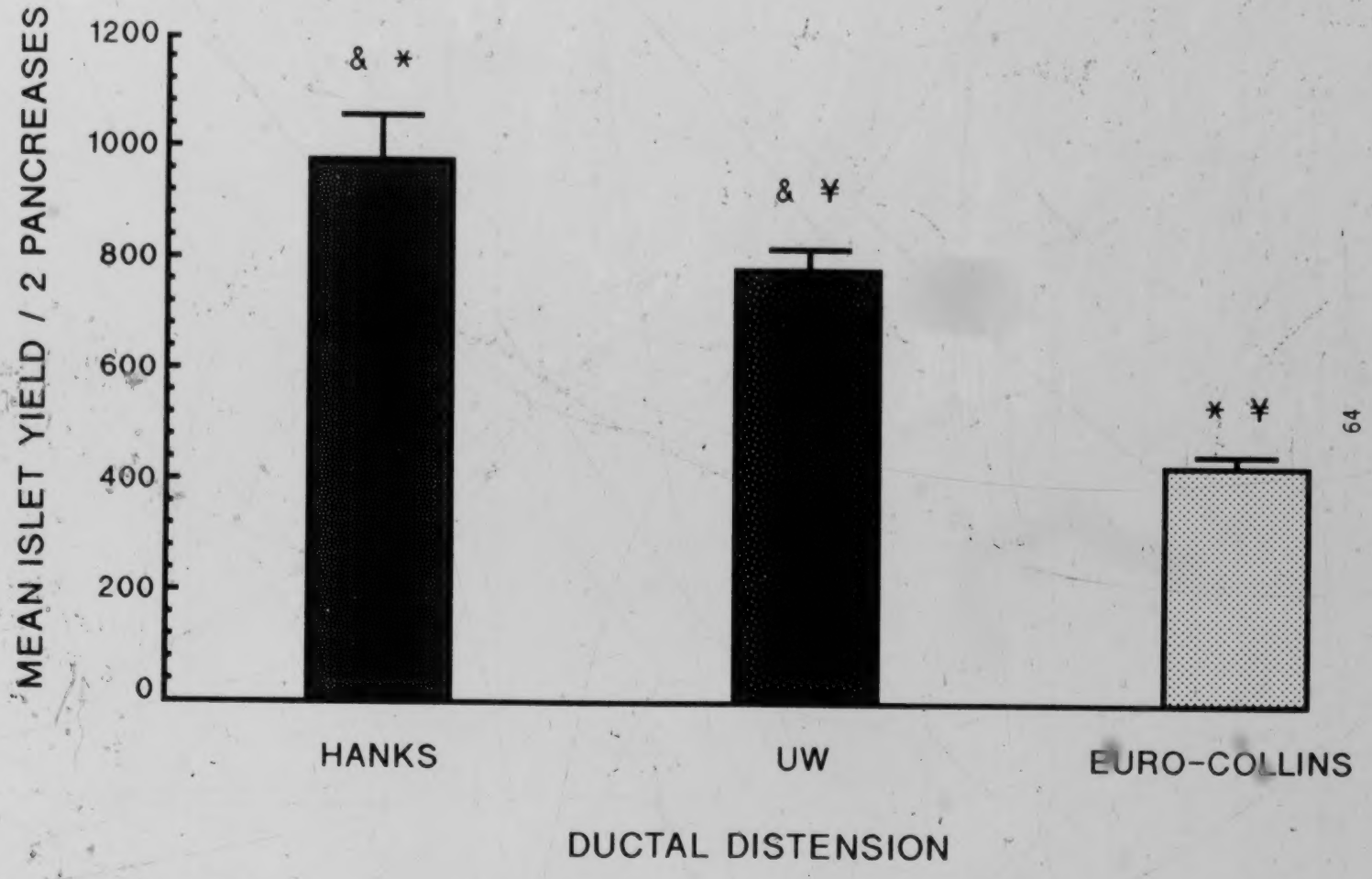

MEAN ISLET YIELD / 2 PANCREASES
1200
1000
800
600
400
200
0
& *
& ¥
* ¥
HANKS
UW
EURO-COLLINS
DUCTAL DISTENSION
64

FIGURE 2:

Mean islet yields following intraabdominal vascular flushing prior to ductal distension were significantly higher for UW solution than for Euro-Collins solution (497.6 ± 26 and 295.4 ± 19.3) following immediate islet isolation. Bars represent +1 standard error of the mean. N = 7 (14 animals) for both experimental groups. * symbol indicates significant differences ($p < 0.05$) in mean islet yield between the two experimental groups.

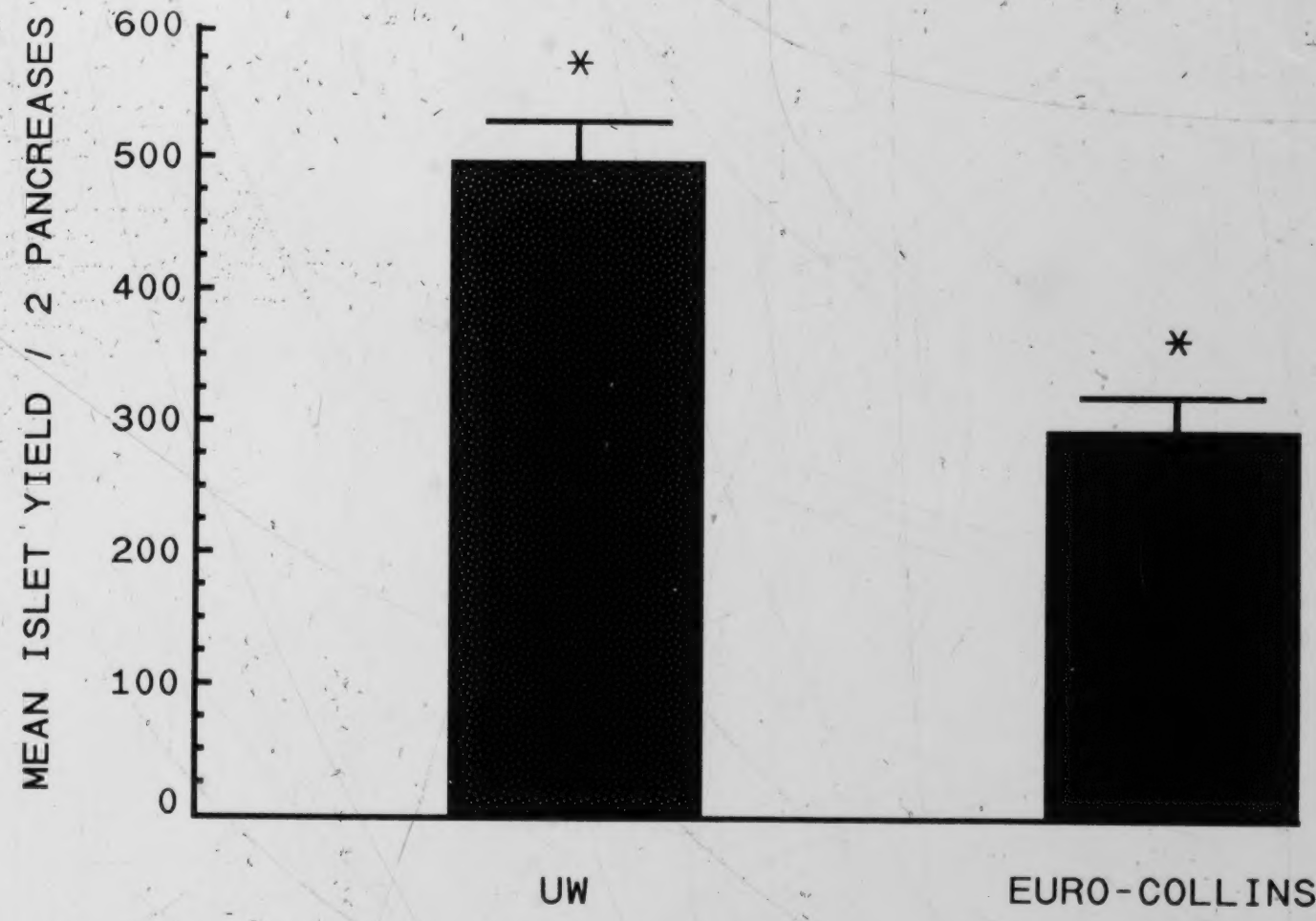

MEAN ISLET YIELD / 2 PANCREASES
600
500
400
300
200
100
0
*
*
UW
EURO-COLLINS
VASCULAR FLUSH AND DUCTAL DISTENSION

FIGURE 3:

Ductal distension using UW solution and storage in UW solution for 24 hours results in the highest islet yields following (318 ± 10.8) as compared to preservation in Euro-Collins solution following ductal distension (7.3 ± 1.6) or preservation for 24 hours in either solution following vascular flush (10.7 ± 1.7 for UW and 6.6 ± 1.5 for Euro-Collins solution). Bars represent +1 standard error of the mean. N = 7 (14 animals) for all of the experimental groups. Bars sharing * symbol indicate statistical differences ($p < 0.05$) in mean islet yields.

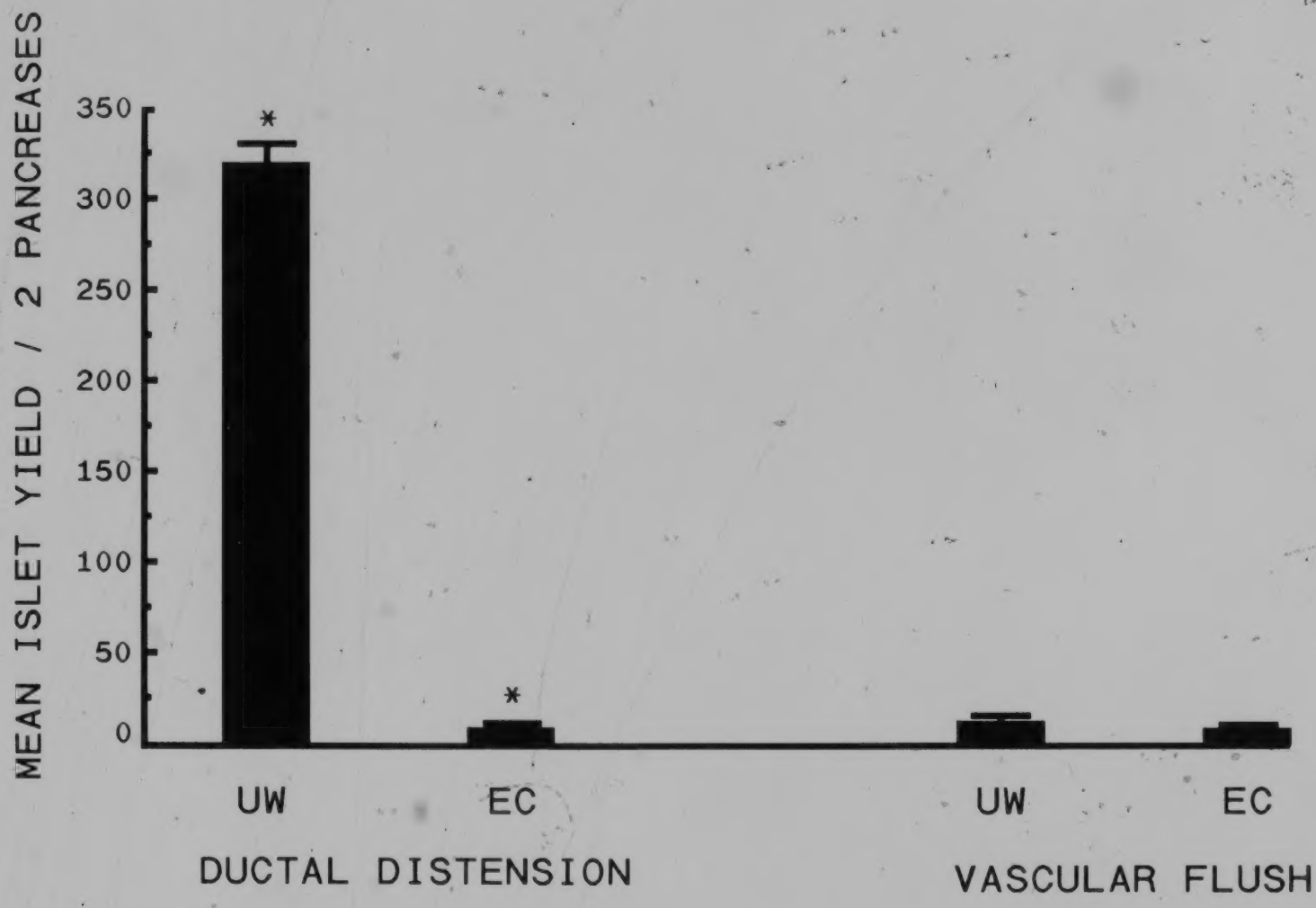

MEAN ISLET YIELD / 2 PANCREASES
350
300
250
200
150
100
50
0
*
*
UW
EC
UW
EC
DUCTAL DISTENSION
VASCULAR FLUSH

FIGURE 4:

Extended hypothermic storage of pancreas at 4°C results in a progressive decrease in the ability to recover islets. Islet isolation following cold storage for 6 hours yielded 464.5 ± 26.5 islets per experiment. Further decreases in islet yields were observed when the preservation interval was extended to 18 hours (383 ± 11.5); 24 hours (318 ± 10.1) and 48 hours (6.1 ± 1). Bars represent +1 standard error of the mean. N = 7 (14 animals) for all of the experimental groups. All preservation groups were statistically significant from the 0 hour control group ($p < 0.05$). In addition, bars sharing the same symbols indicate significant differences ($p < 0.05$) in mean islet yields.

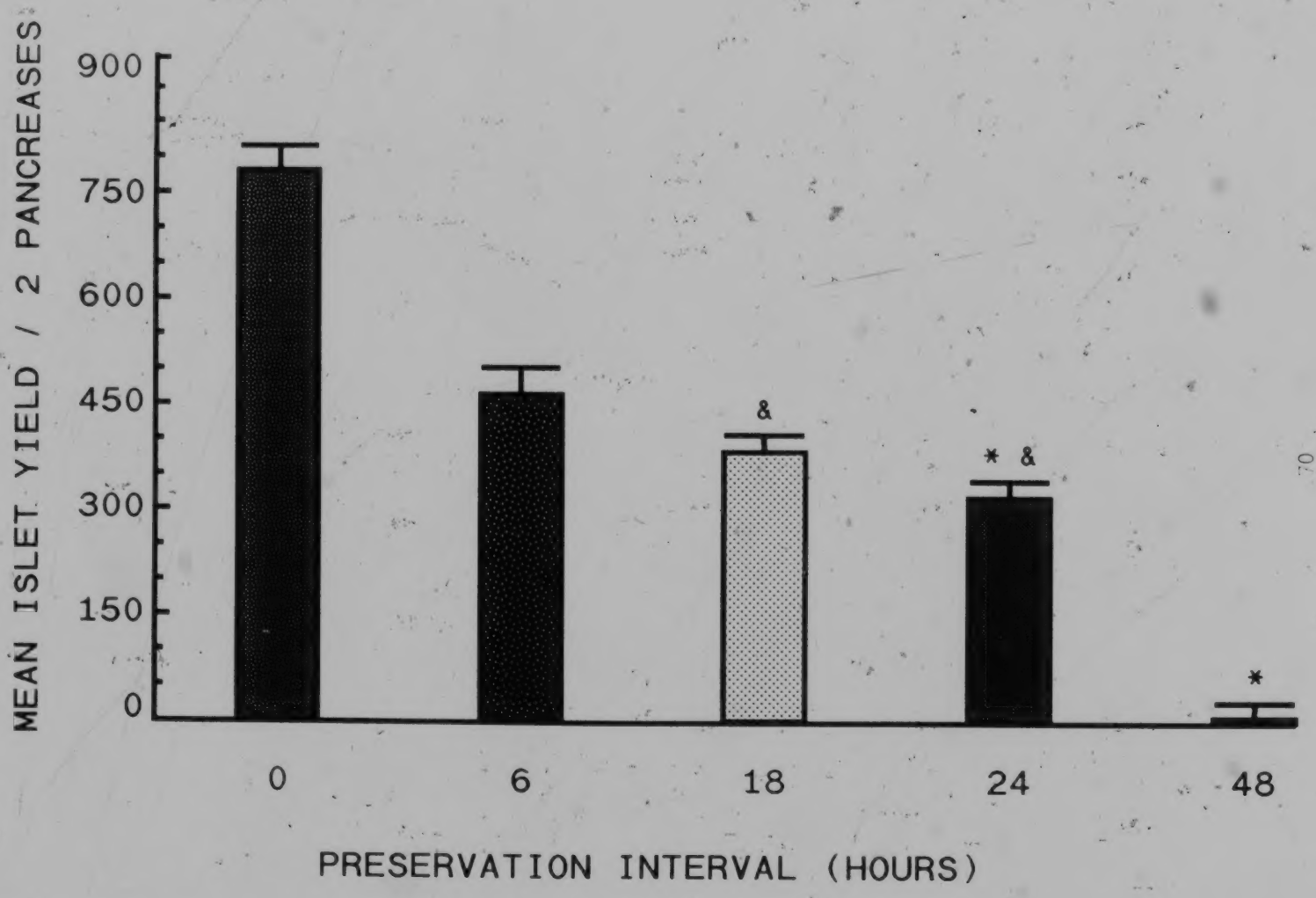

MEAN ISLET YIELD / 2 PANCREASES
900
750
600
450
300
150
0
&
* &
*
0
6
18
24
48
PRESERVATION INTERVAL (HOURS)

FIGURE 5:

Mean islet yields (2 pancreases) without preservation (control) and following 24 hour cold storage at various preservation temperatures (4°C = 318 ± 10.1; 7°C = 410.9 ± 4.4; 10°C = 466.7 ± 28.5; and 15°C = 44.6 ± 3.9). N = 7 (14 animals) for all preservation groups and for the control group. Bars represent +1 standard error of the mean. All preservation group values are statistically significantly less ($p < 0.0001$) than the control value. In addition, bars sharing the same symbols indicate significant difference ($p < 0.05$) in mean islet yields.

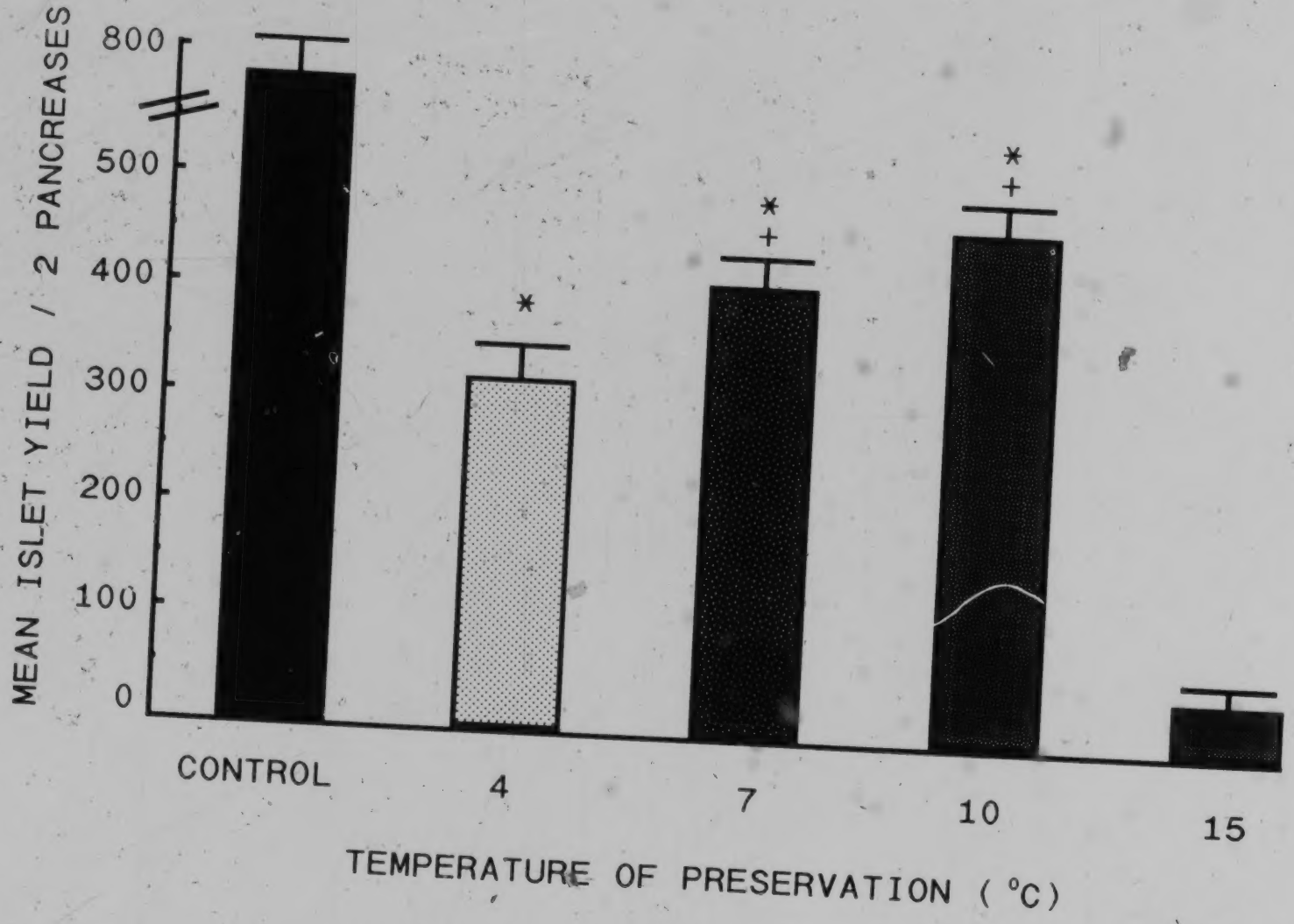
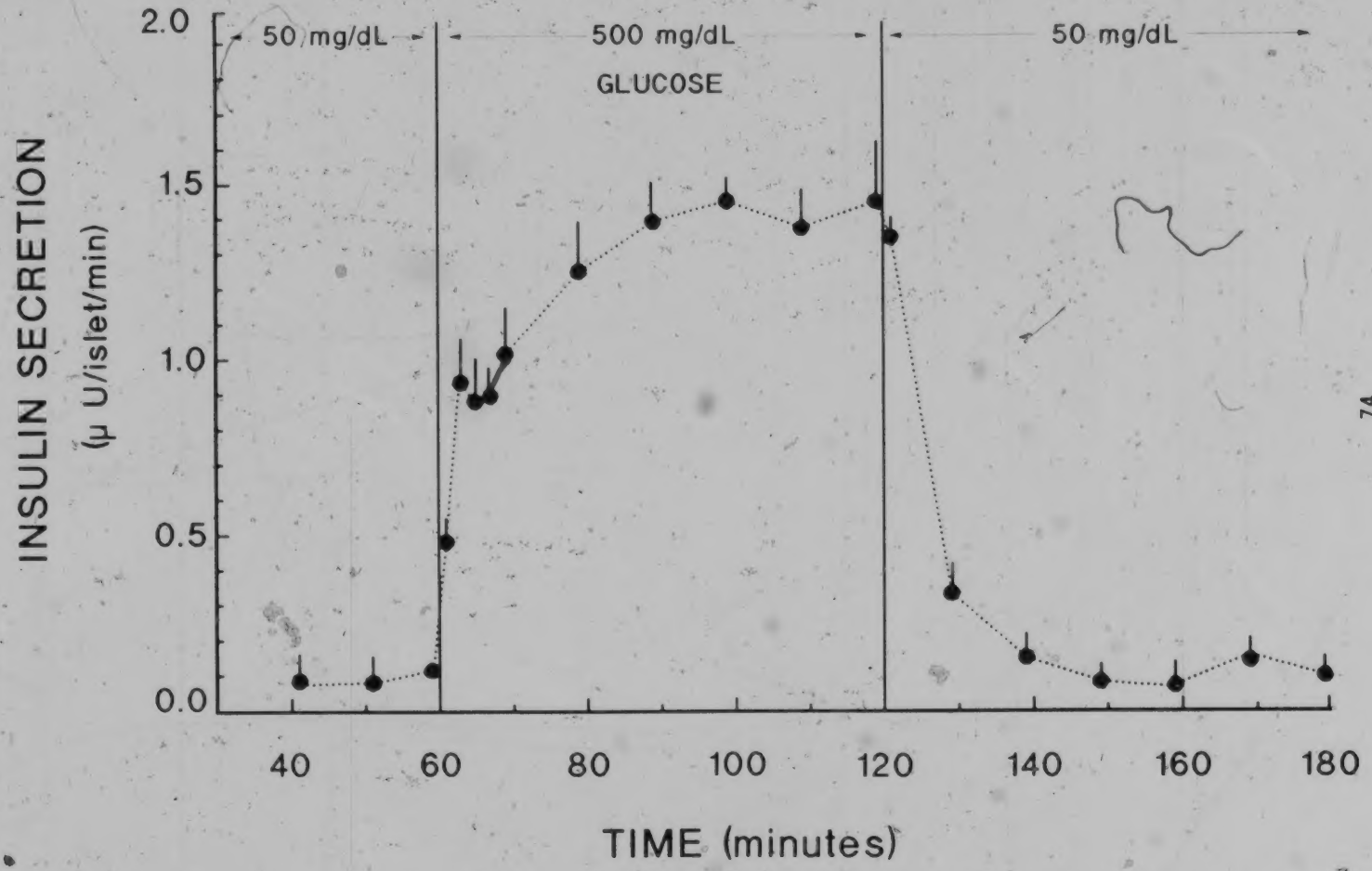

MEAN ISLET YIELD / 2 PANCREASES
800
500
400
300
200
100
0
CONTROL
4
7
10
15
TEMPERATURE OF PRESERVATION (°C)

FIGURE 6:

In vitro insulin response of freshly isolated islets to low (50 md/dL) and high (500 mg/dL) glucose stimulations during perifusion. The mean insulin secretion rate (μ U/islet/min) from 28 perifusion chambers has been plotted with the vertical bars representing +1 standard error of the mean.


50 mg/dL
500 mg/dL
GLUCOSE
50 mg/dL
INSULIN SECRETION
(μ U/islet/min)
2.0
1.5
1.0
0.5
0.0
40
60
80
100
120
140
160
180
TIME (minutes)

74

FIGURE 7:

In vitro insulin response of isolated islets to low (50 mg/dL) and high (500 mg/dL) glucose stimulations during perifusion following 24 hours of hypothermic preservation. The mean insulin secretion rate (μ U/islet/min) from 21 perifusion chambers for the 4°C group, and 24 chambers from the 7 and 10°C groups, has been plotted with the vertical bars representing +1 standard error of the mean.

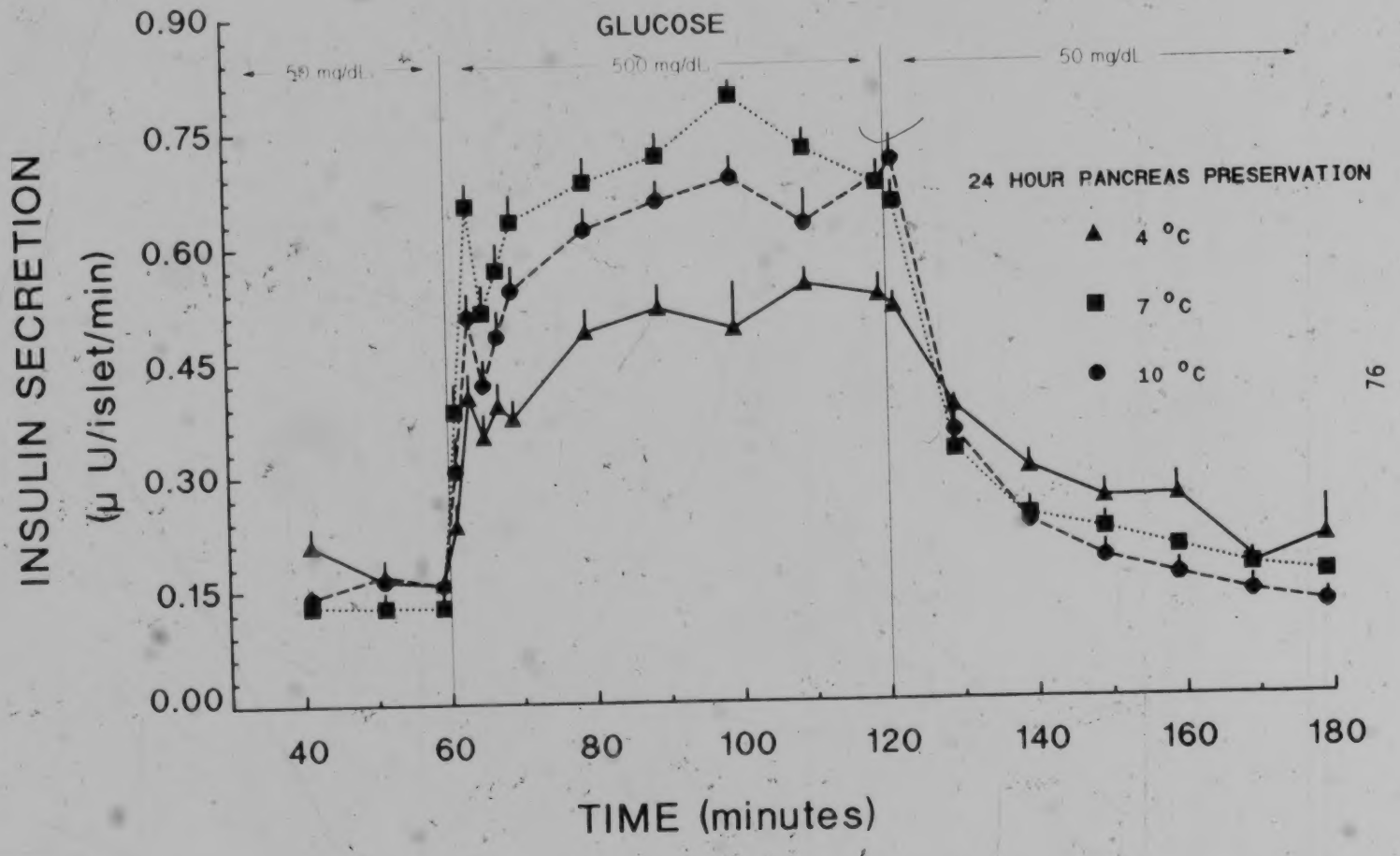

GLUCOSE
50 mg/dL
500 mg/dL
50 mg/dL
24 HOUR PANCREAS PRESERVATION
4 °C
7 °C
10 °C
INSULIN SECRETION
(μ U/islet/min)
0.90
0.75
0.60
0.45
0.30
0.15
0.00
40
60
80
100
120
140
160
180
TIME (minutes)
76

FIGURE 8:

Calculation of the Mean Insulin Secretion Rate (MISR) and the Islet Preservation Index (IPI) from glucose perifusions. "A" and "C" each represents the area under the curve for the pre- and post-glucose stimulation period respectively. Whereas, "B" represents the area under the curve for the high glucose stimulation period of the perifusion.

The MISR and IPI are calculated using the equations found in Table 1.

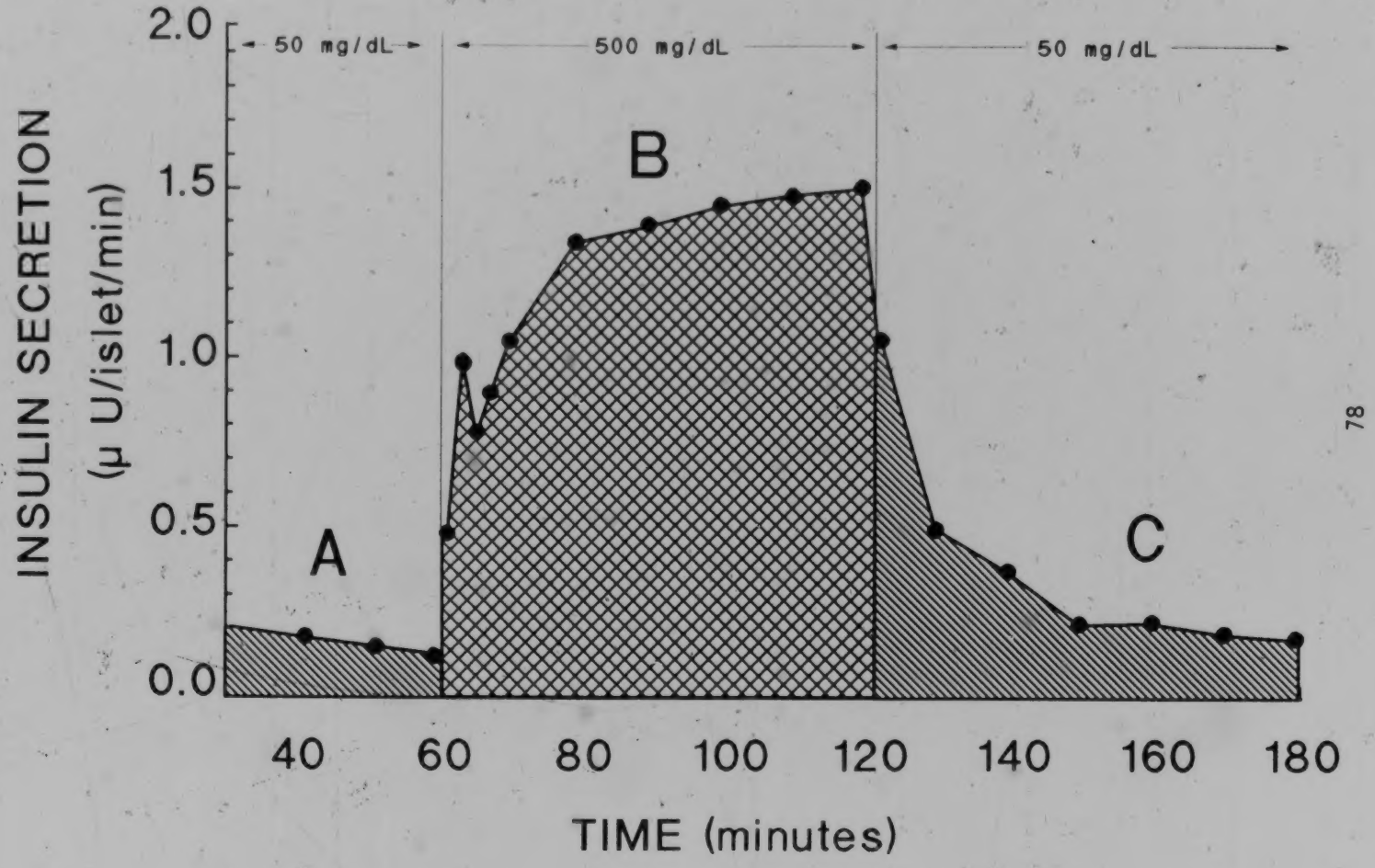

50 mg/dL
500 mg/dL
50 mg/dL
A
B
C
INSULIN SECRETION
(μ U/islet/min)
2.0
1.5
1.0
0.5
0.0
40
60
80
100
120
140
160
180
TIME (minutes)
78

FIGURE 9:

Perifusion graph comparing secretory capabilities of islets preserved at 7°C and islets isolated after 24 hour whole pancreas preservation at 7°C. The mean insulin secretion rate, (μ U/islet/min) from 24 perifusion chambers from both groups, has been plotted with the vertical bars representing +1 standard error of the mean.

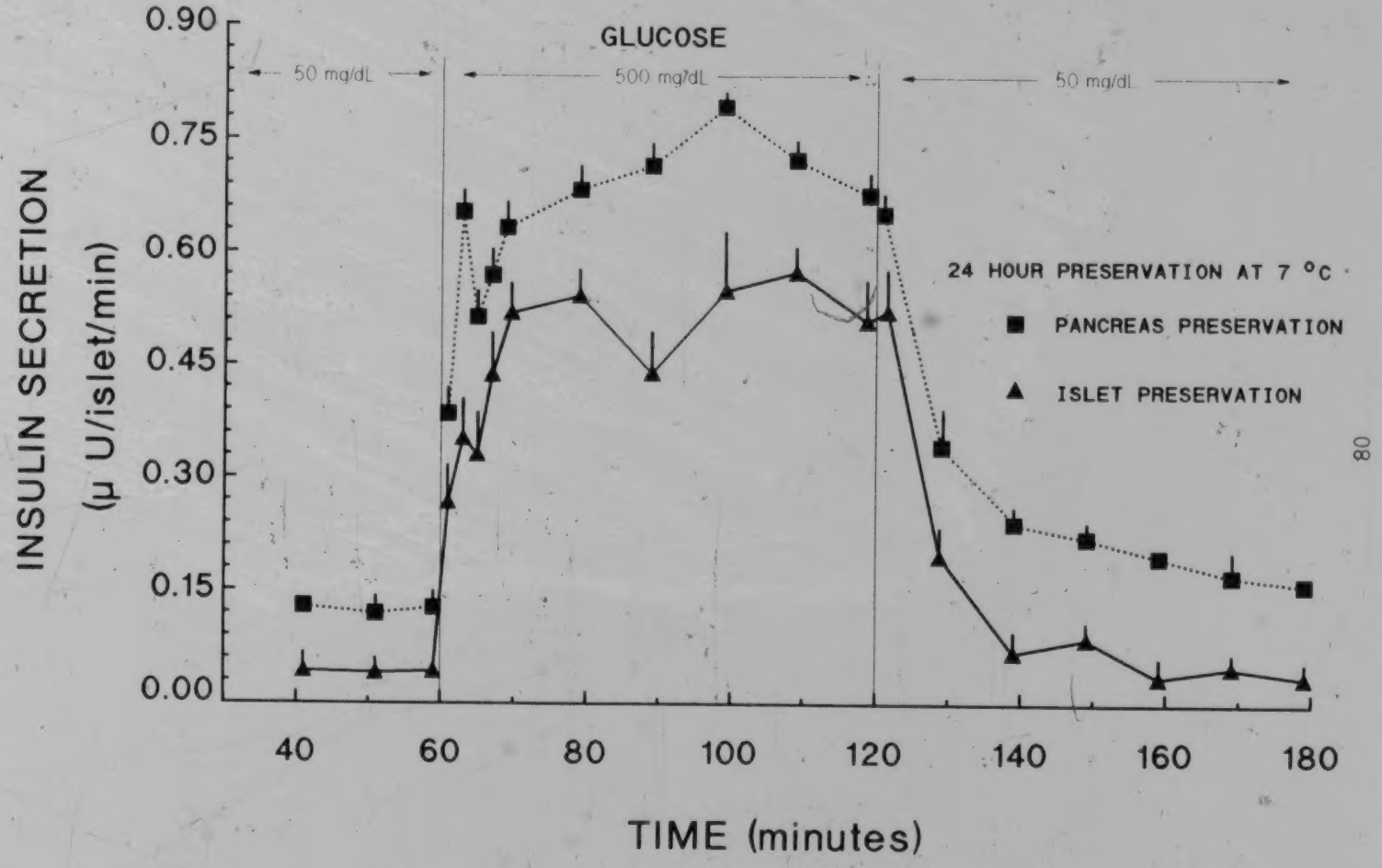

GLUCOSE
50 mg/dL
500 mg/dL
50 mg/dL
0.90
0.75
0.60
0.45
0.30
0.15
0.00
INSULIN SECRETION
(μ U/islet/min)
40
60
80
100
120
140
160
180
TIME (minutes)
24 HOUR PRESERVATION AT 7 °C
PANCREAS PRESERVATION
ISLET PRESERVATION
80

TABLE 1: Calculations for the Determination of Mean Insulin Secretion Rate (MISR) and Islet Preservation Index (IPI).

Mean Insulin Secretion Rate:

$$MISR = \frac{B}{A+C}$$

Where A, B, and C are areas under the response curve in pre-glucose, high glucose stimulation, and post-glucose periods.

Example:

$$B = \frac{\text{Sum of Insulin Output During High Glucose Stimulation}}{(\#\text{ of Samples})\ (\#\text{ of Islets Perifused})}$$

$$B = \frac{496.1}{(11)\ (100)}$$

$$B = 0.451$$

$$MISR = \frac{0.451}{0.1785 + 0.2476}$$

$$MISR = 1.06$$

Islet Preservation Index:

$$IPI = \frac{MISR \times \text{Mean Islet Yields Following Preservation}}{\text{Mean Islet Yields (Control)}}$$

Example: 24 Hour Preservation at 4°C

$$IPI = \frac{1.77 \times 318.0}{780.3}$$

$$IPI = 0.722$$

TABLE 2: Pancreatic Islet Count, Mean Insulin Secretion Rate (MISR) and Islet Preservation Index (IPI) following 24 Hour Preservation at Varying Hypothermic Temperatures.

	CONTROL	24 HOUR PRESERVATION GROUPS			
		4°C	7°C	10°C	15°C
MEAN ISLET YIELD	780.3	318.0	410.9	466.7	44.6
(± sem)	(21.8)	(10.1)	(24.4)	(28.5)	(3.9)
% OF CONTROL	-----	40.8	52.7	59.8	5.7
MISR	4.97	1.77[a,b]	2.73[a,c]	2.42[b,c]	---
(± sem)	(0.223)	(0.071)	(0.102)	(0.096)	
% OF CONTROL	-----	35.6	54.9	48.7	---
IPI	-----	0.722[d,e]	1.44[d]	1.45[e]	---
(± sem)		(0.03)	(0.05)	(0.06)	

Statistical differences ($p < 0.05$) for pairwise comparisons of MISR or IPI are indicated by the same letter ("a" superscript denotes significant difference of MISR between 4 and 7°C).

TABLE 3: Mean Insulin Secretion Rate (MISR) Following 24 Hour Whole Pancreas Preservation or Isolated Islet Preservation at 7°C.

	CONTROL	PRESERVATION GROUPS	
		WHOLE PANCREAS	ISOLATED ISLETS
MISR	4.97[a]	2.73[a,b]	4.91[b]
(± sem)	(0.223)	(0.102)	(0.181)
% OF CONTROL	-----	54.9	98.8

Statistical differences ($p < 0.05$) for pairwise comparisons of MISR are indicated by the same letter ("a" superscript denotes significant difference between the control and the 7°C pancreas group).

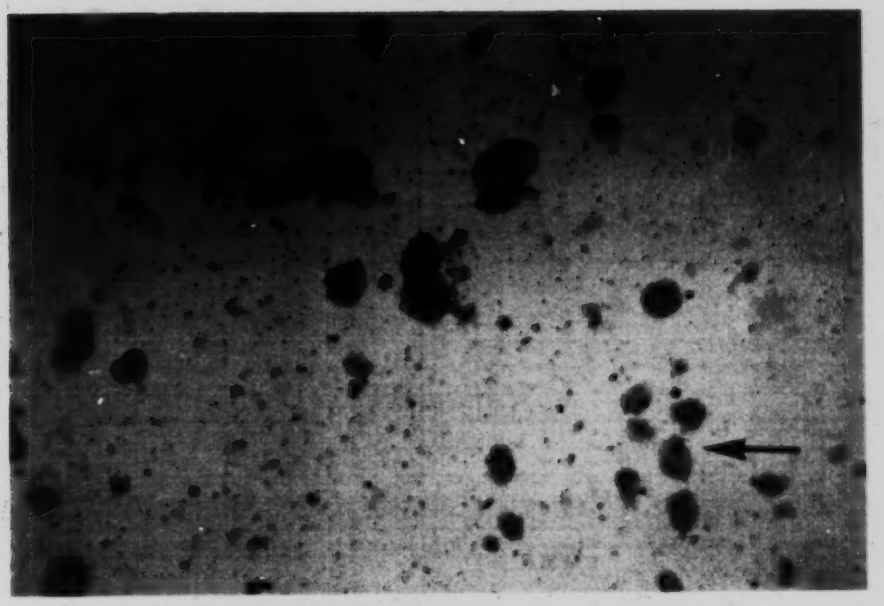


PLATE I:

Dithizone Stained Freshly Isolated Islets of Langerhans

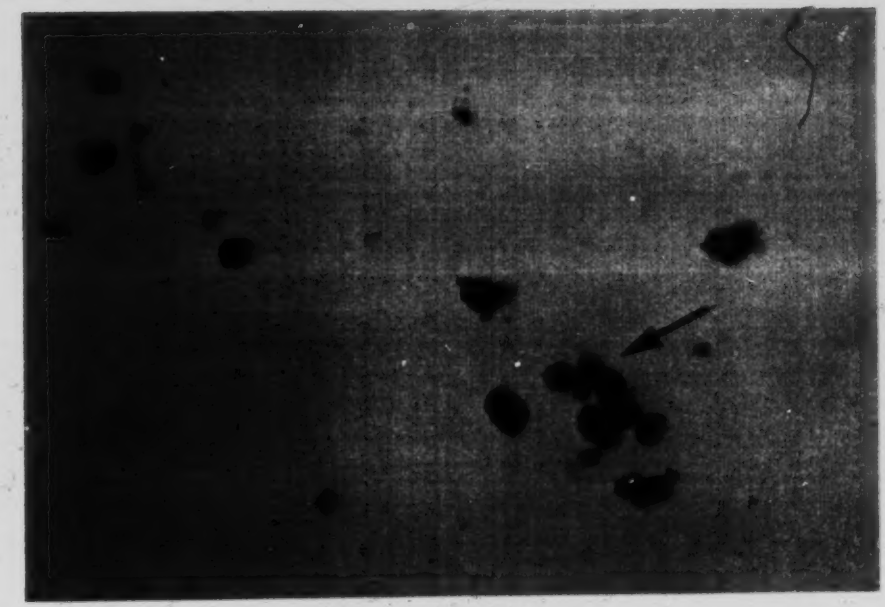


PLATE II:

Dithizone Stained Isolated Islets of Langerhans Following 24 Hour Hypothermic Preservation

PLATE III:
Dithizone Stained Post-Ficoll Exocrine Pellet
Following 24 Hour Hypothermic Preservation

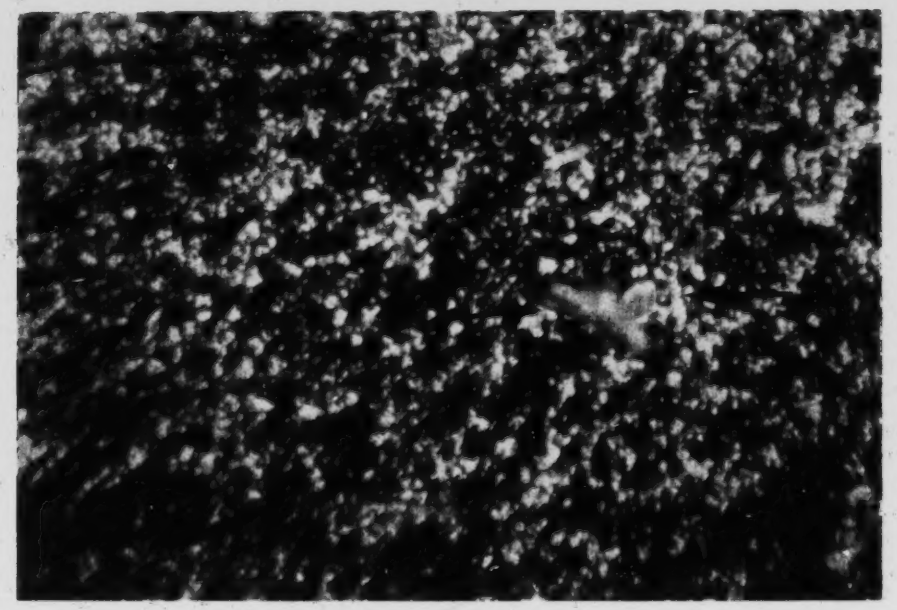


PLATE IV:
Dithizone Stained Post-Ficoll Exocrine Pellet
with Attached Exocrine Fragment Following
24 Hour Hypothermic Preservation

PLATE V:

Dithizone Stained Post-Ficoll Exocrine Pellet From Freshly Isolated Pancreatic Tissue

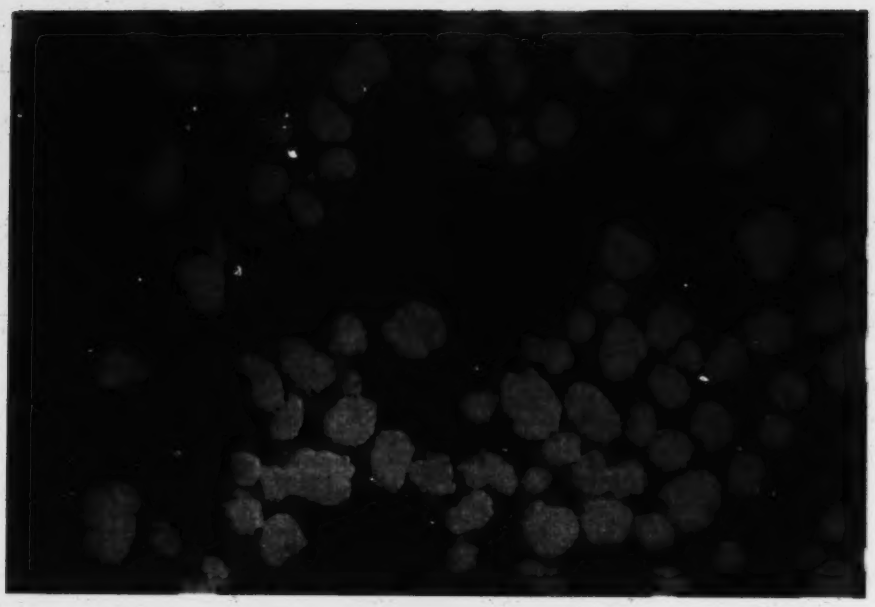


PLATE VI:

Freshly Isolated Islets of Langerhans as Identified Using the Green Gel Technique

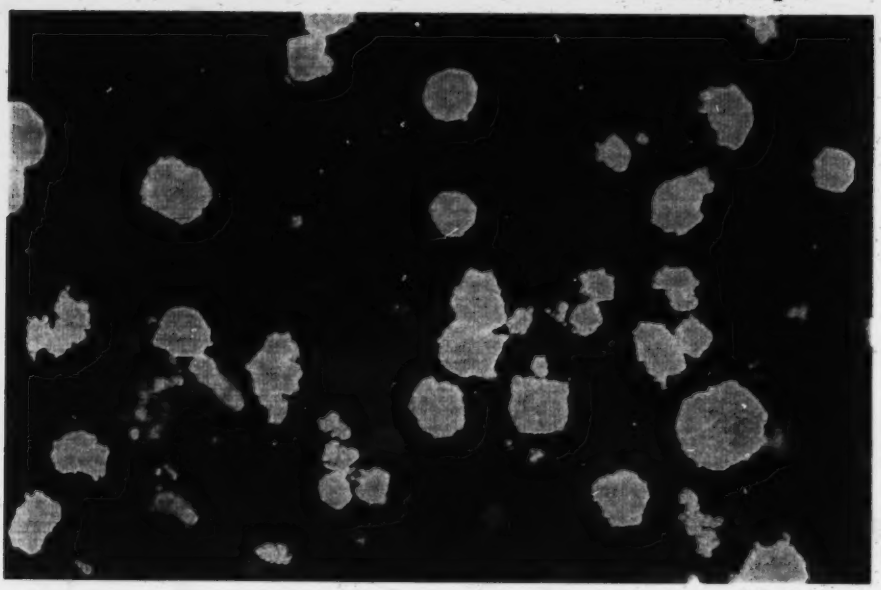


PLATE VII:

Isolated Islets of Langerhans Following 24 Hour Hypothermic Pancreas Preservation as Identified Using Green Gel Technique

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APPENDIX A:

HANKS BALANCED SALT SOLUTION

Component	Amount in 1 Liter (g/L)
Inorganic Salts:	
$CaCl_2$	0.14
KCl	0.40
KH_2PO_4	0.06
$MgCl_2.6\ H_2O$	0.10
$MgSO_4.7\ H_2O$	0.10
NaCl	8.00
$Na_2HPO_4.7\ H2O$	0.09
$NaHCO_3$	0.35
Other Components:	
D-Glucose	1.00
Phenol Red (Sodium)	0.01

Solution Specifics:

pH at 25°C	7.40 ± 0.30
Osmolality	284.0 ± 5.0 % mOsm/Liter

APPENDIX B:

COMPOSITION OF UNIVERSITY OF WISCONSIN COLD STORAGE SOLUTION

Substance	Amount in 1 Liter (g/L)
K^+-Lactobionate	37.73
KH_2PO_4	3.40
$MgSO_4$	1.23
Raffinose	17.83
Adenosine	1.34
Glutathione	0.92
Insulin (24 UI/mg)	0.004
Penicillin/Streptomycin (25,000 units/mL 25,000 mcg/mL)	4.5 mL
Dexamethasone	0.008
Allopurinol	0.136
Hydroxyethyl Starch	50.0

Solution Specifics:

pH at 25°C	7.40 ± 0.30
Osmolality	320.0 ± 5.0 % mOsm/Liter

APPENDIX C:

COMPOSITION OF EURO-COLLINS ORGAN PRESERVATION SOLUTION

Component:	Amount in 1 Liter (g/L)
KH_2PO_4	2.05
K_2HPO_4	7.40
KCl	1.12
$NaHCO_3$	0.84
D-Glucose	35.0

Solution Specifics:

pH at 25°C	7.40 ± 0.30
Osmolality	355.0 ± 5.0 % mOsm/Liter

The solution was either purchased from Travenol Laboratories Inc. (Deerfield, IL) or made from chemicals purchased from Sigma (St. Louis, MO).

APPENDIX D:

FICOLL GRADIENT FOR RAT

Stock Solution: Dissolve 54.0 g of Ficoll 400 DL (Sigma) in 162 mL of Hanks' Balanced Salt Solution (HBSS) (Gibco) with 25 mM Hepes (Gibco)

FICOLL CONCENTRATIONS	STOCK	HBSS
25 %	50.0 mL	-----
23 %	45.60 mL	4.40 mL
20 %	40.26 mL	9.76 mL
11 %	21.06 mL	28.96 mL

APPENDIX E:

MEDIUM 199 SOLUTION

Component	Amount in 1 Liter (mg/L)
Inorganic Salts:	
$CaCl_2$	200
$Fe(NO_3)_3 \cdot 9\ H_2O$	0.72
KCl	400
$MgSO_4 \cdot 7\ H_2O$	200
NaCl	6100
$NaHCO_3$	2200
$NaH_2PO_4 \cdot H_2O$	140
Other Components:	
Adenine Sulfate	10
Adenosinetriphosphate (disodium salt)	1.0
Adenylic acid	0.20
Cholesterol	0.20
Deoxyribose	0.50
D-Glucose	1000
Glutathione (reduced)	0.50
Guanine HCl	0.30
HEPES	5958
Hypoxanthine	0.30
Phenol red	200
Ribose	0.50
Sodium acetate	50
Thymine	0.30
Tween 80	20
Uracil	3.0
Xanthine (sodium)	3.0
Amino Acids:	
DL-Alanine	50
L-Arginine HCl	70
DL-Aspartic acid	60
L-Cysteine $HCl \cdot H_2O$	0.11
L-Cystine	20
DL-Glutamic acid $\cdot H_2O$	150
L-Glutamine	100
Glycine	50

Component	Amount in 1 Liter (mg/L)
Amino acids: continued	
L-Histidine HCl H_2O	21.88
L-Hydroxyproline	10
DL-Isoleucine	40
L-Lysine HCl	70
DL-Methionine	30
DL-Phenylalanine	50
L-Proline	40
DL-Serine	50
DL-Threonine	60
DL-Trypophan	20
L-Tryosine	40
Vitamins:	
Ascorbic acid	0.05
α-Tocopherol phosphate (disodium salt)	0.01
d-Biotin	0.01
Calciferol	0.10
D-Ca pantothenate	0.01
Choline chloride	0.50
Folic acid	0.01
i-Inositol	0.05
Menadione	0.01
Niacin	0.025
Niacinamide	0.025
Para-aminobenzoic acid	0.05
Pyridoxal HCl	0.025
Pyridoxine HCl	0.025
Riboflavin	0.01
Thiamine HCl	0.01
Vitamin A (acetate)	0.14

Solution Specifics:

pH at 25°C	6.80 ± 0.30
Osmolality	256.0 ± 5.0 % mOsm/Liter

APPENDIX F:

RPMI 1640 SOLUTION

Formula # 80-5013

Component	Amount in 1 Liter (mg/L)
Inorganic Salts:	
$Ca(NO_3)_2$ 4 H_2O	100
KCl	400
$MgSO_4$	48.84
NaCl	5850
Na_2HPO_4 (anhyd.)	800
Other Components:	
Glutathione (reduced)	1.0
HEPES	5957.5
Phenol red	5.0
Amino Acids:	
L-Arginine (free base)	200
L-Asparagine	50
L-Aspartic acid	20
L-Cystine 2HCl	65.15
L-Glutamic acid	20
L-Glutamine	300
Glycine	10
L-Hystidine (free base)	15
L-Hydroxyproline	20
L-Isoleucine (allo free)	50
L-Leucine (methionine free)	50
L-Lycine HCl	40
L-Methionine	15
L-Phenylalanine	15
L-Proline (hydroxy free)	20
L-Serine	30
L-Threonine (allo free)	20
L-Tryptophan	5
L-Tyrosine (disodium salt)	28.83
L-Valine	20
Vitamins:	
Biotin	0.20
D-Ca pantothenate	0.25

Component	Amount in 1 Liter (mg/L)
Vitamins: continued	
Choline chloride	1.0
Folic acid	1.0
i-Inositol	35
Nicotinamide	1.0
Para-aminobenzoic acid	1.0
Pyridoxine HCl	1.0
Riboflavin	0.02
Thiamine HCl	1.0
Vitamin B_{12}	0.005

Solution Specifics:

pH at 25°C	7.0 ± 0.30
Osmolality	260.0 ± 5 % Osm/Liter (without $NaHCO_3$)
	292.0 ± 5 % mOsm/Liter (with $NaHCO_3$)

APPENDIX G:

PERIFUSION SAMPLING SEQUENCE

Sample: #	Time: (minute)
********** 50 mg/dL ********** Glucose	
1	41-43
2	51-53
3	59-61
********** 500 mg/dL ********** Glucose	
4	61-63
5	63-65
6	65-67
7	67-69
8	69-71
9	79-81
10	89-91
11	99-101
12	109-111
13	119-121
********** 50 mg/dL ********** Glucose	
14	121-123
15	129-131
16	139-141
17	149-151
18	159-161
19	169-171
20	179-181

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